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(71) Applicant *(for all designated States except US)*: **BIOGEN, INC.** [US/US]; 14 Cambridge Center, Cambridge, MA 02142 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **LING, Leona, E.** [US/US]; 45 Fells Road, Winchester, MA 01890 (US). **SANICOLA-NADEL, Michele** [US/US]; 4 Maple Road, Winchester, MA 01890 (US).

(74) Agents: VINCENT, Matthew, P. et al.; Ropes & Gray, Patent Group, One International Place, Boston, MA 02110 (US).

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(54) Title: ANGIOGENESIS-MODULATING COMPOSITIONS AND USES

Alignment of N-terminal fragments of Human Hedgehog Proteins

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        1
Indian  CGPGRVVGSR RRPPRK-LVP LAYKQFSPNV PEKTLGASGR YEGKIARSSE
Sonic   CGPGRGFG-K RRHPKK-LTP LAYKQFIPNV AEKTLGASGR YEGKISRNSE
Desert  CGPGRGPVGR RRYARKQLVP LLYKQFVPGV PERTLGASGP AEGRVARGSE

        51
Indian  RFKELTPNYN PDIIFKDEEN TGADRLMTQR CKDRLNSLAI SVMNQWPGVK
Sonic   RFKELTPNYN PDIIFKDEEN TGADRLMTQR CKDKLNALAI SVMNQWPGVK
Desert  RFRDLVPNYN PDIIFKDEEN SGADRLMTER CKERVNALAI AVMNMWPGVR

        101
Indian  LRVTEGWDED GHHSEESLHY EGRAVDITTS DRDRNKYGLL ARLAVEAGFD
Sonic   LRVTEGWDED GHHSEESLHY EGRAVDITTS DRDRSKYGML ARLAVEAGFD
Desert  LRVTEGWDED GHHAQDSLHY EGRALDITTS DRDRNKYGLL ARLAVEAGFD

        151
Indian  WVYYESKAHV HCSVKSEHSA AAKTGG      SEQ ID NO: 23
Sonic   WVYYESKAHI HCSVKAENSV AAKSGG      SEQ ID NO. 24
Desert  WVYYESRNHV HVSVKADNSL AVRAGG      SEQ ID NO. 25
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Gap(s), indicated by -, added to facilitate alignment

(57) Abstract: Hedgehog agonists and antagonists can be used to regulate angiogenesis, and have utility in treating tissue repair and cancer, and to prevent angiogenesis driven pathologies.

WO 01/98344 A2

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

ANGIOGENESIS-MODULATING COMPOSITIONS AND USES

Background Art

Hedgehog proteins act as morphogens in a wide variety of tissues during embryonic development (Ingham, 1995; Perrimon, 1995; Johnson and Tabin, 1997; Hammerschmidt et al., 1997). Vertebrate hedgehogs are crucial to a number of epithelial-mesenchymal inductive interactions during neuronal development, limb development, lung, bone, hair follicle and gut formation (Ericson et al., 1995; Roberts et al., 1995; Apelqvist et al., 1997; Ericson et al., 1997; Hammerschmidt et al., 1997; Johnson and Tabin, 1995; Pepicelli et al., 1998; Litingtung et al., 1998; Roberts et al., 1998; Dodd et al., 1998; Dockter, 2000). Mammalian hedgehog genes consist of sonic, indian and desert which are highly conserved between species (Zardoya, 1996). Sonic hedgehog (shh) is expressed widely during development and sonic null mice are embryonic lethal with multiple defects beginning early to midgestation (Bitgood and McMahon, 1995; Chiang et al., 1996; Litingtung et al., 1998; St-Jacques et al., 1998). Indian hedgehog (ihh) is expressed less widely and indian null mice survive till late gestation. However, Ihh null mice exhibit severe stunting of skeletal growth which correlates to the role of Ihh in regulating bone growth plate (St-Jacques et al., 1999; Karp et al., 2000). Desert hedgehog (dhh) is the most restricted in expression and Dhh null mice are viable, but as expected from the expression pattern, male gonads do not develop completely and the peripheral nerves develop in a disorganized fashion (Bitgood et al., 1996; Parmantier et al., 1999).

Hedgehog signalling occurs through the interaction of hedgehog protein with the hedgehog receptor, patched (Ptc) and this interaction's modulation of the co-receptor smoothened (Smo). The mammalian genome contains 2 patched genes, ptcl and ptc2, both of which encode 12 transmembrane proteins containing a sterol sensing domain (Motoyama et al, 1998; Carpenter et al, 1998). The interaction of Hh and Ptc inactivates the repression of smoothened (Smo), a 7 transmembrane protein which then leads to activation of fused (Fu), a serine-threonine kinase, and the disassociation of a

transcription factor, Gli, from the microtuble-associated Fu-Gli-Su(fu) complex. The uncomplexed Gli protein is transported to the nucleus where it activates downstream target genes of the hedgehog pathway including the ptc1 and gli1 genes (Ding et al., 1999; Murone et al, 1999a; Murone et al, 1999b; Pearse et al., 1999; Stone et al., 1999; Hynes et al, 2000).

Hedgehog genes have so far not been implicated directly in embryonic or adult angiogenesis. No vascular defects have been reported in shh, ihh or dhh knockout mice. However, we show here that cells in the adult vasculature both express ptc1 and can respond to exogenous hedgehog and, more importantly, hedgehog is able to induce robust neovascularization in the corneal pocket model of angiogenesis. The angiogenic response to hedgehog appears to occur through the activation of mesenchymal cells to produce VEGFs and Angiopoietins.

Angiogenesis, the process of sprouting new blood vessels from existing vasculature and arteriogenesis, the remodeling of small vessels into larger conduit vessels are both physiologically important aspects of vascular growth in adult tissues (Klagsbrun and D'Amore, 1991; Folkman and Shing, 1992; Beck and D'Amore, 1997; Yancopoulos et al., 1998; Buschman and Schaper, 2000). These processes of vascular growth are required for beneficial processes such as tissue repair, wound healing, recovery from tissue ischemia and menstrual cycling. They are also required for the development of pathological conditions such as the growth of neoplasias, diabetic retinopathy, rheumatoid arthritis, psoriasis, certain forms of macular degeneration, and certain inflammatory pathologies (Cherrington et al., 2000).

The ability to stimulate vascular growth has potential utility for treatment of ischemia-induced pathologies such as myocardial infarction, coronary artery disease, peripheral vascular disease, and stroke. The sprouting of new vessels and/or the expansion of small vessels in ischemic tissues prevents ischemic tissue death and induces tissue repair. Certain growth factors such as those in the vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) families are able to stimulate vascular growth by acting on endothelial cells to induce angiogenesis. Other factors

have also been shown to have angiogenic and arteriogenic activities such as MCPI (Buschman and Schaper, 2000) and angiopoietins. In preclinical models of myocardial infarction, both FGFs and VEGFs have been able to improve myocardial revascularization and function (Yanagisawa-Miwa et al, 1992; Battler et al., 1993; Harada et al., 1994; Banai et al., 1994; Unger et al., 1994; Mesri et al., 1995; Pearlman et al., 1995; Landau et al, 1995; Lazarous et al., 1996; Engler, 1996; Magovern et al., 1997; Shou et al., 1997). Also in models of peripheral vascular disease, VEGF and other angiogenic factors are able to induce angiogenesis and improve vascular perfusion of the ischemic limb (Majesky, 2000; Takeshita et al, 1996 and 1994; Rivard et al., 1998 and 1999, Isner et al, 1996).

A number of these factors are also implicated in vascular growth in pathological conditions such as tumor expansion, diabetic retinopathy and rhematoid arthritis. The inhibiton of vascular growth in these contexts has also shown beneficial effects in preclinical animal models (Klohs and Hamby, 1999; Zhu and Witte, 1999; Cherrington et al., 2000). For example, inhibition of angiogenesis by blocking vascular endothelial growth factor or its receptor has resulted in inhibition of tumor growth and in retinopathy (Fong et al., 1999; Wood et al., 2000; Ozaki et al., 2000). Also, the development of pathological pannus tissue in rheumatoid arthritis involves angiogenesis and can be blocked by inhibitors of angiogenesis (Peacock et al., 1995; Storgard et al., 1999).

Thus, the induction of angiogenesis and vascular growth is beneficial for tissue repair and would healing whereas inhibition of angiogenic growth factors can prevent angiogenesis driven pathologies. It would be useful to develop novel therapeutics that modulate angiogenesis.

Summary Of The Invention

Hedgehog proteins are angiogenic growth factors which can have utility in treating tissue repair and ischemia and that inhibition of the hedgehog proteins and the hedgehog pathway can prevent angiogenesis driven pathologies.

Brief Description of Drawings

Figure 1 : Alignment of N-terminal fragments of Human Hedgehog Proteins

Figure 2: Consensus sequence of a hedgehog protein suitable for use in developing the conjugated proteins of the invention, antagonist, where "Xaa" indicates amino acids that differ between the Sonic, Indian and Desert hedgehog proteins.

Detailed Description Of The Invention

The present invention relates to the use of hedgehog protein, DNA, or other hedgehog therapeutic as an agent to induce the growth of new blood vessels, ie angiogenesis, arteriogenesis or vascular growth in adult tissues where the induction of angiogenesis has therapeutic value. The present invention also relates to the use of inhibitors of hedgehog protein or signaling to prevent angiogenesis contributing to pathological conditions such as neoplasia (tumors and gliomas), diabetic retinopathy, rheumatoid arthritis, osteroarthritis, macular degeneration, psoriasis, ulcerative colitis, Chrohn's disease, and inflammation.

All references cited in the Detailed Description are incorporated herein by references, unless stipulated otherwise. The following terms are used herein:

I. Definitions

"Angiogenesis" is defined as any alteration of an existing vascular bed or the formation of new vasculature which benefits tissue perfusion. This includes the formation of new vessels by sprouting of endothelial cells from existing blood vessels or the remodeling of existing vessels to alter size, maturity. direction or flow properties to improve blood perfusion of tissue.

Mesenchymal cells are defined as cells of mesenchymal origin including fibroblasts, stromal cells, smooth muscle cells, skeletal muscle cells, cells of osteogenic origin such as chondrocytes, cells of hemaeopoietic origin such as monocytes, macrophages, lymphocytes, granulocytes and cells of adipose origin such as adipocytes.

A hedgehog therapeutic, whether it is a hedgehog angonist or hedgehog antagonist is said to have "therapeutic efficacy" in modulating angiogenesis and an amount of the therapeutic is said to be a "angiogenic modulatory amount", if administration of that amount of the therapeutic is sufficient to cause a significant modulation (i.e., increase or decrease) in angiogenic activity when administered to a subject (e.g., an animal model or human patient) needing modulation of angiogenesis.

As used herein, a hedgehog therapeutic of the invention is an "agonist" if it "modulates" hedgehog biological activity (i.e., elicits, allows and/or enhances hedgehog biological activity). For the purposes of the invention an agonist also refers to an agent, e.g., a polypeptide such as an hedgehog or patched or a small organic molecule which can elicit, allow and/or enhance hedgehog and/or patched-mediated binding or which can otherwise modulate hedgehog and/or patched function, e.g., by activating hedgehog-ligand mediated hedgehog signal transduction. Such an agonist of the hedgehog/patched interaction is an agent which has one or more of the following properties: (1) it coats, or binds to, a hedgehog protein associated with an extracellular matrix, e.g., heparin, heparin proteoglycans, collagen, fibronectin, vitronectin, thrombospondin, or on the surface of a hedgehog bearing or secreting cell with sufficient specificity to modulate a hedgehog-ligand/hedgehog receptor interaction, e.g., the hedgehog/patched-smoothened interaction; (2) it coats, or binds to, a hedgehog on the surface of a hedgehog- bearing or secreting cell with sufficient specificity to modify, and preferably to modulate, transduction of a hedgehog-mediated signal e.g., hedgehog/patched-smoothened - mediated signaling; (3) it coats, or binds to, a hedgehog receptor or co-receptor, (e.g., patched, smoothened or a heparin proteoglycan) in or on cells with sufficient specificity to modulate the hedgehog/patched-smoothened interaction; (4) it coats, or binds to, a hedgehog receptor (e.g., patched or smoothened) in or on cells with sufficient specificity to modify, and preferably to modulate, transduction of hedgehog receptor mediated hedgehog signaling, e.g., patched, smoothened, fused or gli-mediated hedgehog signaling.

In preferred embodiments an agonist has one or both of properties 1 and 2. In other preferred embodiments the agonist has one or both of properties 3 and 4. Moreover, more than one agonist can be administered to a patient, e.g., an agent which binds to hedgehog can be combined with an agent which binds to patched. Moreover, a hedgehog therapeutic is an "agonist" if it modulates angiogenesis in such a way as to enhance, elicit, accelerate or increase angiogenesis, regardless of the mode of action of such therapeutic.

As used herein, a hedgehog therapeutic is an "antagonist" if it de-activates the hedgehog receptor or inhibits its activity or inhibits activity of the hedgehog protein. Such an antagonist may additionally have one or more of the following properties: (1) it coats, or binds to, a hedgehog protein on the surface of a hedgehog bearing or secreting cell with sufficient specificity to de-activate or inhibit a hedgehog-ligand/hedgehog interaction, e.g., the hedgehog/patched interaction; (2) it coats, or binds to, a hedgehog protein on the surface of a hedgehog- bearing or secreting cell with sufficient specificity to modify, and preferably to de-activate or inhibit, transduction of a hedgehog-mediated signal e.g., hedgehog/patched, smoothened, fused, or gli -mediated signaling; (3) it coats, or binds to, a hedgehog receptor or coreceptor (e.g., patched or smoothened) in or on cells with sufficient specificity to de-activate or inhibit the hedgehog /patched interaction; (4) it coats, or binds to, a hedgehog receptor or co-receptor (e.g., patched or smoothened) in or on cells with sufficient specificity to modify, and preferably to de-activate or inhibit transduction of hedgehog receptor mediated hedgehog signaling, e.g., patched-mediated hedgehog signaling. In preferred embodiments an antagonist has one or both of properties 1 and 2. In other preferred embodiments the antagonist has one or both of properties 3 and 4. Moreover, more than one antagonist can be administered to a patient, e.g., an agent which binds to hedgehog can be combined with an agent which binds to patched. Moreover, a hedgehog therapeutic is an "antagonist' if it modulates angiogenesis in such a way as to inhibit, decelerate, reverse or otherwise slow angiogenesis, regardless of the mode of action of such therapeutic. For example, antagonist molecules may be antibody homologs (defined below), certain fragments of

hedgehog, or small organic molecules that may be administered and modulate hedgehog binding sites on cells.

As discussed herein, the hedgehog therapeutics (i.e., antagonists or agonists) that can be linked or otherwise conjugated to, for instance, an antibody homolog such as an immunoglobulin or fragment thereof are not limited to a particular type or structure of hedgehog or patched or other molecule so that, for purposes of the invention, any agent capable of forming a chimeric protein and capable of effectively modulating hedgehog is considered to be an equivalent of the therapeutics used in the examples herein.

As used herein, the term “antibody homolog” includes intact antibodies consisting of immunoglobulin light and heavy chains linked via disulfide bonds. The term “antibody homolog” is also intended to encompass a hedgehog therapeutic comprising one or more polypeptides selected from immunoglobulin light chains, immunoglobulin heavy chains and antigen-binding fragments thereof which are capable of binding to one or more antigens (i.e., hedgehog or patched). The component polypeptides of an antibody homolog composed of more than one polypeptide may optionally be disulfide-bound or otherwise covalently crosslinked. Accordingly, therefore, "antibody homologs" include intact immunoglobulins of types IgA, IgG, IgE, IgD, IgM (as well as subtypes thereof), wherein the light chains of the immunoglobulin may be of types kappa or lambda or portions of intact antibodies that retain antigen-binding specificity, for example, Fab fragments, Fab' fragments, F(ab')2 fragments, F(v) fragments, heavy chain monomers or dimers, light chain monomers or dimers, dimers consisting of one heavy and one light chain, and the like.

As used herein, a “humanized antibody homolog” is an antibody homolog, produced by recombinant DNA technology, in which some or all of the amino acids of a human immunoglobulin light or heavy chain that are not required for antigen binding have been substituted for the corresponding amino acids from a nonhuman mammalian immunoglobulin light or heavy chain. A “human antibody homolog” is an antibody homolog in which all the amino acids of an immunoglobulin light or heavy chain

(regardless of whether or not they are required for antigen binding) are derived from a human source.

"amino acid"- a monomeric unit of a peptide, polypeptide, or protein. There are twenty amino acids found in naturally occurring peptides, polypeptides and proteins, all of which are L-isomers. The term also includes analogs of the amino acids and D-isomers of the protein amino acids and their analogs.

A hedgehog therapeutic has "biological activity" if it has at least one of the following properties: (i) it has the ability to bind to its receptor, patched or it encodes, upon expression, a polypeptide that has this characteristic; and/or (ii) it may induce alkaline phosphatase activity in C3H10T1/2 cells. The hedgehog therapeutic protein meeting this functional test of "biological activity" may meet the hedgehog consensus criteria as defined herein in Figure 2 (SEQ 1D NO: 26). This term "biological activity" includes antagonists and agonists.

The term "bioavailability" refers to the ability of a compound to be absorbed by the body after administration. For instance, a first compound has greater bioavailability than a second compound if, when both are administered in equal amounts, the first compound is absorbed into the blood to a greater extent than the second compound.

The term "chimeric" hedgehog therapeutic is a generic term referring to constructs X-A, where "X" is a polypeptide having the amino acid sequence or portion thereof, consisting of the amino acid sequence of a hedgehog protein and "A" is at least part of a polypeptide other than hedgehog. "A" may include a linker sequence (as defined below) and may be attached to either, or both, of the N- or C-terminii of the hedgehog moiety. Chimeric hedgehog therapeutics of the invention therefore include compounds in which the various moieties are chemically cross-linked or covalently "fused" (as defined below).

As used herein, the term "covalently coupled" means that the specified moieties of the hedgehog therapeutic are either directly covalently bonded to one another, or else are indirectly covalently joined to one another through an intervening moiety or moieties, such as a bridge, spacer, or linkage moiety or moieties. The intervening moiety

or moieties are called a "coupling group". The term "conjugated" is used interchangeably with "covalently coupled".

"expression control sequence"- a sequence of polynucleotides that controls and regulates expression of genes when operatively linked to those genes.

"expression vector"- a polynucleotide, such as a DNA plasmid or phage (among other common examples) which allows expression of at least one gene when the expression vector is introduced into a host cell. The vector may, or may not, be able to replicate in a cell.

The phrase "extracellular signaling protein" means any protein that is either secreted from a cell, or is associated with the cell membrane, and upon binding to the receptor for that protein on a target cell, triggers a response in the target cell.

"functional equivalent" of an amino acid residue is (i) an amino acid having similar reactive properties as the amino acid residue that was replaced by the functional equivalent; (ii) an amino acid of a ligand of a polypeptide of the invention, the amino acid having similar properties as the amino acid residue that was replaced by the functional equivalent; (iii) a non-amino acid molecule having similar properties as the amino acid residue that was replaced by the functional equivalent.

A first polynucleotide encoding hedgehog protein is "functionally equivalent" compared with a second polynucleotide encoding hedgehog protein if it satisfies at least one of the following conditions:

(a) the "functional equivalent" is a first polynucleotide that hybridizes to the second polynucleotide under standard hybridization conditions and/or is degenerate to the first polynucleotide sequence. Most preferably, it encodes a mutant hedgehog having the activity of an hedgehog therapeutic;

(b) the "functional equivalent" is a first polynucleotide that codes on expression for an amino acid sequence encoded by the second polynucleotide.

The term "hedgehog therapeutic" includes, but is not limited to, the agonist and/or antagonist agents listed herein as well as their functional equivalents. As used herein, the term "functional equivalent" therefore refers to, for example, an hedgehog

protein or a polynucleotide encoding the hedgehog protein that has the same or an improved beneficial effect on the mammalian recipient as the hedgehog of which it is deemed a functional equivalent. As will be appreciated by one of ordinary skill in the art, a functionally equivalent protein can be produced by recombinant techniques, e.g., by expressing a "functionally equivalent DNA". Accordingly, the instant invention embraces hedgehog therapeutics encoded by naturally-occurring DNAs, as well as by non-naturally-occurring DNAs which encode the same protein as encoded by the naturally-occurring DNA. Due to the degeneracy of the nucleotide coding sequences, other polynucleotides may be used to encode hedgehog protein. These include all, or portions of the above sequences which are altered by the substitution of different codons that encode the same amino acid residue within the sequence, thus producing a silent change. Such altered sequences are regarded as equivalents of these sequences. For example, Phe (F) is coded for by two codons, TTC or TTT, Tyr (Y) is coded for by TAC or TAT and His (H) is coded for by CAC or CAT. On the other hand, Trp (W) is coded for by a single codon, TGG. Accordingly, it will be appreciated that for a given DNA sequence encoding a particular hedgehog there will be many DNA degenerate sequences that will code for it. These degenerate DNA sequences are considered within the scope of this invention.

The term "fusion" or "fusion protein" is a species of chimeric hedgehog therapeutic and refers to a co-linear, covalent linkage of two or more proteins or fragments thereof via their individual peptide backbones, most preferably through genetic expression of a polynucleotide molecule encoding those proteins. It is preferred that the proteins or fragments thereof are from different sources (e.g., a 'chimeric' protein). Thus, preferred fusion therapeutics include an hedgehog protein or fragment covalently linked to a second moiety that is not a hedgehog protein. In certain embodiments, the non-hedgehog moiety may be a protein having a domain or region which is homologous to a member of the immunoglobulin gene superfamily. Members of this superfamily inlcude class I and class II major histocompatability antigens, CD4 and T cell receptor chains. Further examples of members of this family and fusion

proteins containing them are found in US 5,565,335 (Genentech), incorporated herein by reference.

Non-hedgehog proteins of this type are useful if they contain one or more amino acid sequences at least 20, 50, 75 or 150 residues in length, that are at least 40% homologous to a sequence of an immunoglobulin constant or variable region. A non-hedgehog protein meeting these requirements is said to possess an "Ig-like domain" which may be an "Ig-like constant domain" or an "Ig-like variable domain". Thus, one embodiment of the present invention is a chimeric hedgehog therapeutic in which the non-hedgehog moiety contains at least one Ig-like domain, or portion thereof.

Other embodiments are possible. Specifically, a "hedgehog/Ig fusion" is a hedgehog therapeutic comprising a biologically active hedgehog molecule of the invention (i.e., Sonic hedgehog), or a biologically active fragment thereof (i.e., the N-terminal portion) linked to an N-terminus of an immunoglobulin chain wherein a portion of the N-terminus of the immunoglobulin is replaced with the hedgehog. A species of hedgehog/Ig fusion is an "hedgehog /Fc fusion" which is a protein comprising an hedgehog molecule of the invention (i.e., hedgehog -) linked to at least a part of the constant domain of an immunoglobulin. Also, the term "fusion protein" means an hedgehog protein chemically linked via a mono- or hetero- functional molecule to a second moiety that is not an hedgehog protein and is made de novo from purified protein as described below. Thus, this invention features a hedgehog therapeutic molecule which includes: (1) a hedgehog moiety, (2) a second peptide, e.g., one which increases solubility or in vivo life time of the hedgehog moiety, e.g., a member of the immunoglobulin super family or fragment or portion thereof, e.g., a portion or a fragment of IgG, e.g., the human IgGl heavy chain constant region, e.g., CH2, CH3, and hinge regions; and a toxin moiety.

"Heterologous promoter"- as used herein is a promoter which is not naturally associated with a gene or a purified nucleic acid.

"Homology" and "identity" each refer to sequence similarity between two polypeptide sequences, and both `homology and 'identity' are used interchangeably in

this disclosure. Homology can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same amino acid residue, then the polypeptides can be referred to as identical at that position; when the equivalent site is occupied by the same amino acid (e.g., identical) or a similar amino acid (e.g., similar in steric and/or electronic nature), then the molecules can be referred to as homologous at that position. A percentage of homology between sequences is a function of the number of matching or homologous positions shared by the sequences. An "unrelated" or "non-homologous" sequence shares less than 40 percent identity, though preferably less than 25 percent identity, with a sequence of the present invention.

For instance, if 6 of 10 of the positions in two sequences are matched or are homologous, then the two sequences are 60% homologous. By way of example, the DNA sequences CTGACT and CAGGTT share 50% homology (3 of the 6 total positions are matched). Generally, a comparison is made when two sequences are aligned to give maximum homology. Such alignment can be provided using, for instance, the method of Needleman et al., J. Mol Biol. 48: 443-453 (1970), implemented conveniently by computer programs described in more detail below. Homologous sequences share identical or similar amino acid residues, where similar residues are conservative substitutions for, or "allowed point mutations" of, corresponding amino acid residues in an aligned reference sequence. In this regard, a "conservative substitution" of a residue in a reference sequence are those substitutions that are physically or functionally similar to the corresponding reference residues, e.g., that have a similar size, shape, electric charge, chemical properties, including the ability to form covalent or hydrogen bonds, or the like. Particularly preferred conservative substitutions are those fulfilling the criteria defined for an "accepted point mutation" in Dayhoff et al., 5: Atlas of Protein Sequence and Structure, 5: Suppl. 3, chapter 22: 354-352, Nat. Biomed. Res. Foundation, Washington, D.C. (1978).

"Percent homology/identity" of two amino acids sequences or two nucleic acid sequences is determined using the alignment algorithm of Karlin and Altschul (Proc.

Nat. Acad. Sci., USA 87: 2264 (1990) as modified in Karlin and Altschul (Proc. Nat. Acad. Sci., USA 90: 5873 (1993). Such an algorithm is incorporated into the NBLAST or XBLAST programs of Altschul et al., J. Mol. Biol. 215: 403 (1990). BLAST searches are performed with the NBLAST program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to a nucleic acid of the invention. BLAST protein searches are performed with the XBLAST program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to a reference polypeptide. To obtain gapped alignments for comparisons, gapped BLAST is used as described in Altschul et al., Nucleic Acids Res., 25: 3389 (1997). When using BLAST and Gapped BLAST, the default parameters of the respective programs (XBLAST and NBLAST) are used. See http://www/ncbi.nlm.nih.gov.

The term "hedgehog N-terminal fragment" may be used interchangeably with "Hedgehog" and refers to the active mature sequence that is proteolytically cleaved from the hedgehog precursor.

The term "hydrophobic" refers to the tendency of chemical moieties with nonpolar atoms to interact with each other rather than water or other polar atoms. Materials that are "hydrophobic" are, for the most part, insoluble in water. Natural products with hydrophobic properties include lipids, fatty acids, phospholipids, sphingolipids, acylglycerols, waxes, sterols, steroids, terpenes, prostaglandins, thromboxanes, leukotrienes, isoprenoids, retenoids, biotin, and hydrophobic amino acids such as tryptophan, phenylalanine, isoleucine, leucine, valine, methionine, alanine, proline, and tyrosine. A chemical moiety is also hydrophobic or has hydrophobic properties if its physical properties are determined by the presence of nonpolar atoms.

The phrase "internal amino acid" means any amino acid in a peptide sequence that is neither the N-terminal amino acid nor the C-terminal amino acid.

"Isolated" (used interchangeably with "substantially pure") when applied to nucleic acid i.e., polynucleotide sequences that encode polypeptides, means an RNA or DNA polynucleotide, portion of genomic polynucleotide, cDNA or synthetic polynucleotide which, by virtue of its origin or manipulation: (i) is not associated with

all of a polynucleotide with which it is associated in nature (e.g., is present in a host cell as an expression vector, or a portion thereof); or (ii) is linked to a nucleic acid or other chemical moiety other than that to which it is linked in nature; or (iii) does not occur in nature. By "isolated" it is further meant a polynucleotide sequence that is: (i) amplified in vitro by, for example, polymerase chain reaction (PCR); (ii) synthesized chemically; (iii) produced recombinantly by cloning; or (iv) purified, as by cleavage and gel separation.

"Isolated" (used interchangeably with "substantially pure") when applied to polypeptides means a polypeptide or a portion thereof which, by virtue of its origin or manipulation: (i) is present in a host cell as the expression product of a portion of an expression vector; or (ii) is linked to a protein or other chemical moiety other than that to which it is linked in nature; or (iii) does not occur in nature, for example, a protein that is chemically manipulated by appending, or adding at least one hydrophobic moiety to the protein so that the protein is in a form not found in nature. By "isolated" it is further meant a protein that is: (i) synthesized chemically; or (ii) expressed in a host cell and purified away from associated and contaminating proteins. The term generally means a polypeptide that has been separated from other proteins and nucleic acids with which it naturally occurs. Preferably, the polypeptide is also separated from substances such as antibodies or gel matrices (polyacrylamide) which are used to purify it.

"multivalent protein complex" refers to a plurality of hedgehog therapeutics (i.e., one or more).

"mutant" is any change in the genetic material of an organism, in particular any change (i.e., deletion, substitution, addition, or alteration) in a wild type polynucleotide sequence or any change in a wild type protein. The term "mutein" is used interchangeably with "mutant".

"N-terminal end" refers to the first amino acid residue (amino acid number 1) of the mature form of a protein.

"N-terminal cysteine" refers to the amino acid number 1 as shown in SEQ ID NOS. 23-26. In certain embodiments of the hedgehog therapeutic, the N-terminal

cysteine has been "modified". The term "modified" in this regard refers to chemical modifications of the N-terminal cysteine such as linkage thereof to another moiety such as a hydrophobic group and/or replacement of the N-terminal cysteine with another moiety, such as a hydrophobic group.

"operatively linked": A polynucleotide sequence (DNA, RNA) is operatively linked to an expression control sequence when the expression control sequence controls and regulates the transcription and translation of that polynucleotide sequence. The term "operatively linked" includes having an appropriate start signal (e.g., ATG) in front of the polynucleotide sequence to be expressed, and maintaining the correct reading frame to permit expression of the polynucleotide sequence under the control of the expression control sequence, and production of the desired polypeptide encoded by the polynucleotide sequence.

"protein" is any polymer consisting essentially of any of the 20 amino acids. Although "polypeptide" is often used in reference to relatively large polypeptides, and "peptide" is often used in reference to small polypeptides, usage of these terms in the art overlaps and is varied. The term "protein" as used herein refers to peptides, proteins and polypeptides, unless otherwise noted.

The terms "peptide(s)", "protein(s)" and "polypeptide(s)" are used interchangeably herein. The terms "polynucleotide sequence" and "nucleotide sequence" are also used interchangeably herein.

"Recombinant," as used herein, means that a protein is derived from recombinant, mammalian expression systems. Since hedgehog is not glycosylated nor contains disulfide bonds, it can be expressed in most prokaryotic and eukaryotic expression systems.

"Spacer" sequence refers to a moiety that may be inserted between an amino acid to be modified with an antibody homolog or fragment and the remainder of the protein. A spacer is designed to provide separation between the modification and the rest of the protein so as to prevent the modification from interfering with protein function and/or

make it easier for the modification to link with an antibody homolog moiety or any other moiety.

Thus, "substantially pure nucleic acid" is a nucleic acid which is not immediately contiguous with one or both of the coding sequences with which it is normally contiguous in the naturally occurring genome of the organism from which the nucleic acid is derived. Substantially pure DNA also includes a recombinant DNA which is part of a hybrid gene encoding additional hedgehog sequences.

The phrase "surface amino acid" means any amino acid that is exposed to solvent when a protein is folded in its native form.

"standard hybridization conditions" refer to salt and temperature conditions substantially equivalent to 0.5 X SSC to about 5 X SSC and 65°C for both hybridization and wash. The term "standard hybridization conditions" as used herein is therefore an operational definition and encompasses a range of hybridization conditions. Nevertheless, for the purposes of this present disclosure "high stringency" conditions include hybridizing with plaque screen buffer (0.2% polyvinylpyrrolidone, 0.2% Ficoll 400; 0.2% bovine serum albumin, 50 mM Tris-HCl (pH 7.5); 1 M NaCl; 0.1% sodium pyrophosphate; 1% SDS); 10% dextran sulfate, and 100 ug/ml denatured, sonicated salmon sperm DNA at 65 ° C for 12-20 hours, and washing with 75 mM NaCl/7.5 mM sodium citrate (0.5 x SSC)/1% SDS at 65°C. "Low stringency" conditions include hybridizing with plaque screen buffer, 10% dextran sulfate and 110 ug/ml denatured, sonicated salmon sperm DNA at 55°C for 12-20 hours, and washing with 300 mM NaCl/30mM sodium citrate (2.0 X SSC)/1% SDS at 55°C. See also Current Protocols in Molecular Biology, John Wiley & Sons, Inc. New York, Sections 6.3.1-6.3.6, (1989).

A "therapeutic composition" as used herein is defined as comprising the therapeutics of the invention and other biologically compatible ingredients. The therapeutic composition may contain excipients such as water, minerals and carriers such as protein.

"wild type" - the naturally-occurring polynucleotide sequence of an exon of a protein, or a portion thereof, or protein sequence, or portion thereof, respectively, as it normally exists in vivo.

Practice of the present invention will employ, unless indicated otherwise, conventional techniques of cell biology, cell culture, molecular biology, microbiology, recombinant DNA, protein chemistry, and immunology, which are within the skill of the art. Such techniques are described in the literature. Unless stipulated otherwise, all references cited in the Detailed Description are incorporated herein by reference.

II. General Properties of Isolated Hedgehog Proteins

Hedgehogs are a family of genes which begin expression early in development and are involved in the morphogenesis of a number of organs in the developing embryo (Ingham, 1995, Perrimon, 1995; Johnson and Tabin, 1995; Hammerschmidt et al., 1997).

However, there is currently no evidence that hedgehogs are directly involved in the development of the mammalian vasculature. Knockouts of each of the mammalian hedgehog genes, sonic (Chiang et al., 1996; Litingtung et al., 1998; St-Jacques et al., 1998), indian (St-Jacques et al., 1999; Karp et al., 2000) and desert (Bitgood et al., 1996; Parmantier et al., 1999) hedgehog have not been reported to have defects in vascular development, but do show defects in tissues where they are known to function in development.

The adult functions of the hedgehog proteins are not well understood. Hedgehog is known to be expressed in adult bone/cartilage, central and peripheral nervous system, kidney, eye and several other tissues (Valentine et al., 1997; Traiffort et al., 1998 and 1999; Iwamoto et al., 1999; Jensen et al., 1997; Parmantier et al., 1999). The adult function of the hedgehog pathway is perhaps best understood in bone and cartilage where it regulates the differentiation of chondrocytes by modulating PTHrp (Iwamoto et al., 1999; Karp et al., 2000). Administration of hedgehog locally in the skin also can induce hair growth in adult animals (Sato et al., 1999; Wang et al., 2000).

The various naturally-occurring hedgehog proteins from which the subject therapeutics can be derived are characterized by a signal peptide, a highly conserved N-terminal region (see Figure 1), and a more divergent C-terminal domain. In addition to signal sequence cleavage in the secretory pathway (Lee, J.J. et al. (1992) Cell 71:33-50; Tabata, T. et al. (1992) Genes Dev. 2635-2645; Chang, D.E. et al. (1994) Development 120:3339-3353), hedgehog precursor proteins naturally undergo an internal autoproteolytic cleavage which depends on conserved sequences in the C-terminal portion (Lee et al. (1994) Science 266:1528-1537; Porter et al. (1995) Nature 374:363-366). This autocleavage leads to a 19 kD N-terminal peptide and a C-terminal peptide of 26-28 kD. The N-terminal peptide stays tightly associated with the surface of cells in which it was synthesized, while the C-terminal peptide is freely diffusible both in vitro and in vivo. Cell surface retention of the N-terminal peptide is dependent on autocleavage, as a truncated form of hedgehog encoded by an RNA which terminates precisely at the normal position of internal cleavage is diffusible in vitro (Porter et al. (1995) supra) and in vivo (Porter, J.A. et al. (1996) Cell 86, 21-34). Biochemical studies have shown that the autoproteolytic cleavage of the hedgehog precursor protein proceeds through an internal thioester intermediate, which subsequently is cleaved in a nucleophilic substitution. It is suggested that the nucleophile is a small lipophilic molecule, more particularly cholesterol, which becomes covalently bound to the C-terminal end of the N-peptide (Porter *et al.* (1996) *supra*), tethering it to the cell surface.

The vertebrate family of hedgehog genes includes at least four members, e.g., paralogs of the single drosophila hedgehog gene (reference). Three of these members, herein referred to as Desert hedgehog (Dhh), Sonic hedgehog (Shh) and Indian hedgehog (Ihh), apparently exist in all vertebrates, including fish, birds, and mammals. A fourth member, herein referred to as tiggie-winkle hedgehog (Thh), appears specific to fish. Isolated hedgehog proteins used in the methods of this invention are naturally occurring or recombinant proteins of the hedgehog family and may be obtainable from either invertebrate or from vertebrate sources (see references below). Members of the vertebrate hedgehog protein family share homology with proteins encoded by the

Drosophila hedgehog (hh) gene (Mohler and Vani, (1992) Development 115, 957-971). Other members continue to be identified.

Mouse and chicken Shh and mouse Ihh genes (see, for example, U.S. Patent 5,789,543) encode glycoproteins which undergo cleavage, yielding an amino terminal fragment of about 20kDa and a carboxy terminal fragment of about 25kDa. The most preferred 20kDa fragment has the consensus sequence SEQ ID NO: 26 which includes the amino acid sequences of SEQ ID NOS: 23-25. Various other fragments that encompass the 20kDa moiety are considered within the presently claimed invention. Publications disclosing these sequences, as well as their chemical and physical properties, include Hall et al., (1995) Nature 378, 212-216; Ekker et al., (1995) Current Biology 5, 944-955; Fan et al., (1995) Cell 81, 457-465, Chang et al., (1994) Development 120, 3339-3353; Echelard et al., (1993) Cell 75, 1414-1430 34-38; PCT Patent Application WO 95/23223 (Jessell, Dodd, Roelink and Edlund); PCT Patent Publication WO 95/18856 (Ingham, McMahon and Tabin). U.S. Patent 5,759,811 lists the Genbank accession numbers of a complete mRNA sequence encoding human Sonic hedgehog; a partial sequence of human Indian hedgehog mRNA, 5' end; and a partial sequence of human Desert hedgehog mRNA. The hedgehog therapeutic compositions of the subject method can be generated by any of a variety of techniques, including purification of naturally occurring proteins, recombinantly produced proteins and synthetic chemistry. Polypeptide forms of the hedgehog therapeutics are preferably derived from vertebrate hedgehog proteins, e.g., have sequences corresponding to naturally occurring hedgehog proteins, or fragments thereof, from vertebrate organisms. However, it will be appreciated that the hedgehog polypeptide can correspond to a hedgehog protein (or fragment thereof) which occurs in any metazoan organism.

The vertebrate family of hedgehog genes includes at least four members, e.g., paralogs of the single drosophila hedgehog gene (SEQ ID No. 19). Three of these members, herein referred to as Desert hedgehog (Dhh), Sonic hedgehog (Shh) and Indian hedgehog (Ihh), apparently exist in all vertebrates, including fish, birds, and mammals. A fourth member, herein referred to as tiggie-winkle hedgehog (Thh),

appears specific to fish. According to the appended sequence listing, (see also Table 1) a chicken Shh polypeptide is encoded by SEQ ID No: 1; a mouse Dhh polypeptide is encoded by SEQ ID No:2; a mouse Ihh polypeptide is encoded by SEQ ID No:3; a mouse Shh polypeptide is encoded by SEQ ID No:4 a zebrafish Shh polypeptide is encoded by SEQ ID No:5; a human Shh polypeptide is encoded by SEQ ID No:6; a human Ihh polypeptide is encoded by SEQ ID No:7; a human Dhh polypeptide is encoded by SEQ ID No. 8; and a zebrafish Thh is encoded by SEQ ID No. 9.

Table 1

Guide to hedgehog sequences in Sequence Listing

	Nucleotide	Amino Acid
Chicken Shh	SEQ ID No. 1	SEQ ID No. 10
Mouse Dhh	SEQ ID No. 2	SEQ ID No. 11
Mouse Ihh	SEQ ID No. 3	SEQ ID No. 12
Mouse Shh	SEQ ID No. 4	SEQ ID No. 13
Zebrafish Shh	SEQ ID No. 5	SEQ ID No. 14
Human Shh	SEQ ID No. 6	SEQ ID No. 15
Human Ihh	SEQ ID No. 7	SEQ ID No. 16
Human Dhh	SEQ ID No. 8	SEQ ID No. 17
zebrafish Thh	SEQ ID No. 9	SEQ ID No. 18
Drosophila HH	SEQ ID No. 19	SEQ ID No. 20

In addition to the sequence variation between the various hedgehog homologs, the hedgehog proteins are apparently present naturally in a number of different forms, including a pro-form, a full-length mature form, and several processed fragments thereof. The pro-form includes an N-terminal signal peptide for directed secretion of the extracellular domain, while the full-length mature form lacks this signal sequence.

As described above, further processing of the mature form occurs in some instances to yield biologically active fragments of the protein. For instance, sonic hedgehog undergoes additional proteolytic processing to yield two peptides of

approximately 19 kDa and 27 kDa, the 19kDa fragment corresponding to an proteolytic N-terminal portion of the mature protein.

In addition to proteolytic fragmentation, the vertebrate hedgehog proteins can also be modified post-translationally, such as by glycosylation and/or addition of lipophilic moieties, such as stents, fatty acids, etc., though bacterially produced (e.g. unmodified) forms of the proteins still maintain certain of the bioactivities of the native protein. Bioactive fragments of hedgehog polypeptides of the present invention have been generated and are described in great detail in, e.g., PCT publications WO 95/18856 and WO 96/17924.

A "hedgehog therapeutic" of the invention is defined in terms of having at least a portion that consists of the consensus amino acid sequence of SEQ ID NO: 26 or at least a portion that consists of SEQ ID NOS: 10-18 or 23-25. The term also means a hedgehog polypeptide, or a functional variant of a hedgehog polypeptide, or homolog of a hedgehog polypeptide, or functional variant, which has biological activity and can modulate angiogenesis.

Members useful in the methods of the invention include any of the naturally-occurring native hedgehog proteins including allelic, phylogenetic counterparts or other variants thereof, whether naturally-sourced or produced chemically including muteins or mutant proteins, as well as recombinant forms and new, active members of the hedgehog family. Particularly useful hedgehog polypeptides have portions that include all or part of SEQ ID NOS: 23-26.

Hedgehog therapeutics may also include polypeptides having an amino acid sequence at least 60%, 80%, 90%, 95%, 98%, or 99% homologous to an amino acid sequence from SEQ ID NOS 10-18 or 23-26. The polypeptide can also include an amino acid sequence essentially the same as an amino acid sequence in SEQ ID NOS: 10-18 or 23-26. The polypeptide is at least 5, 10, 20, 50, 100, or 150 amino acids in length and includes at least 5, preferably at least 10, more preferably at least 20, most preferably at least 50, 100, or 150 contiguous amino acids from SEQ ID NOS: 10-18 or 23-26.

Polypeptides of the invention include those which arise as a result of the existence of multiple genes, alternative transcription events, alternative RNA splicing events, and alternative translational and posttranslational events. The polypeptide can be made entirely by synthetic means or can be expressed in systems, e.g., cultured cells, which result in substantially the same posttranslational modifications present when the protein is expressed in a native cell, or in systems which result in the omission of posttranslational modifications present when expressed in a native cell.

Moreover, mutagenesis can be used to create modified hh polypeptides, e.g., for such purposes as enhancing therapeutic or prophylactic efficacy, or stability (e.g., ex vivo shelf life and resistance to proteolytic degradation in vivo). Such modified peptides can be produced, for instance, by amino acid substitution, deletion, or addition. Modified hedgehog polypeptides can also include those with altered post-translational processing relative to a naturally occurring hedgehog protein, e.g., altered glycosylation, cholesterolization, prenylation and the like.

In one embodiment, a hedgehog therapeutic is a hedgehog polypeptide with one or more of the following characteristics:

(i) it has at least 30, 40, 42, 50, 60, 70, 80, 90 or 95% sequence identity with amino acids of SEQ ID NOS: 23-26;

(ii) it has a cysteine or a functional equivalent as the N-terminal end;

(iii) it may induce alkaline phosphatase activity in C3H10T1/2 cells;

(iv) it has an overall sequence identity of at least 50%, preferably at least 60%, more preferably at least 70, 80, 90, or 95%, with a polypeptide of SEQ ID NOS: 10-18;

(v) it can be isolated from natural sources such as mammalian cells;

(vi) it can bind or interact with patched; and

(vii) it may be modified at at least one amino acid residue by a polyalkylene glycol polymer attached to the residue or, optionally, via a linker molecule to the amino acid residue.

Preferred nucleic acids encode a polypeptide comprising an amino acid sequence at least 60% homologous or identical, more preferably 70% homologous or identical, and most preferably 80% homologous or identical with an amino acid sequence selected from the group consisting of SEQ ID NOS: 10-18 or 23-26. Nucleic acids which encode polypeptides at least about 90%, more preferably at least about 95%, and most preferably at least about 98-99% homology or identity with an amino acid sequence represented in one of SEQ ID Nos: 23-26 are also within the scope of the invention.

In another embodiment, the hedgehog therapeutic is a polypeptide encodable by a nucleotide sequence that hybridizes under stringent conditions to a hedgehog coding sequence represented in one or more of SEQ ID NOS: 1-9, 19 or 23-26.

Preferred nucleic acids encode a hedgehog polypeptide comprising an amino acid sequence at least 60% homologous, more preferably 70% homologous and most preferably 80% homologous with an amino acid sequence selected from the group consisting of SEQ ID Nos:8-14. Nucleic acids which encode polypeptides at least about 90%, more preferably at least about 95%, and most preferably at least about 98-99% homology with an amino acid sequence represented in one of SEQ ID Nos: l0-18 or 20 are also within the scope of the invention.

Hedgehog therapeutics, in addition to native hedgehog proteins, are at least 60% homologous, more preferably 70% homologous and most preferably 80% homologous with an amino acid sequence represented by any of SEQ ID Nos: 10-18 or 20. Polypeptides which are at least 90%, more preferably at least 95%, and most preferably at least about 98-99% homologous with a sequence selected from the group consisting of SEQ ID Nos: 10-18 or 20 are also within the scope of the invention.

With respect to fragments of hedgehog polypeptide, preferred hedgehogs moieties include at least 50 amino acid residues of a hedgehog polypeptide, more preferably at least 100, and even more preferably at least 150.

Another preferred hedgehog polypeptide which can be included in the hedgehog therapeutic is an N-terminal fragment of the mature protein having a molecular weight of approximately 19 kDa.

Preferred human hedgehog proteins include N-terminal fragments corresponding approximately to residues 24-197 of SEQ ID No. 15, 28-202 of SEQ ID No. 16, and 23-198 of SEQ ID No. 17. By "corresponding approximately" it is meant that the sequence of interest is at most 20 amino acid residues different in length to the reference sequence, though more preferably at most 5, 10 or 15 amino acid different in length.

Still other preferred hedgehog therapeutics include an amino acid sequence represented by the formula A-B wherein: (i) A represents all or the portion of the amino acid sequence designated by residues 24-193 of SEQ ID No: 15; and B represents at least one amino acid residue of the amino acid sequence designated by residues 194-250 of SEQ ID No: 15; (ii) A represents all or the portion of the amino acid sequence designated by residues 25-193 of SEQ ID No: 13; and B represents at least one amino acid residue of the amino acid sequence designated by residues 194-250 of SEQ ID No: 13; (iii) A represents all or the portion of the amino acid sequence designated by residues 23-193 of SEQ ID No: 11; and B represents at least one amino acid residue of the amino acid sequence designated by residues 194-250 of SEQ ID No: 1 l; (iv) A represents all or the portion of the amino acid sequence designated by residues 28-197 of SEQ ID No: 12; and B represents at least one amino acid residue of the amino acid sequence designated by residues 198-250 of SEQ ID No: 12; (v) A represents all or the portion of the amino acid sequence designated by residues 29-197 of SEQ ID No: 16; and B represents at least one amino acid residue of the amino acid sequence designated by residues 198-250 of SEQ ID No: 16; or (vi) A represents all or the portion of the amino acid sequence designated by residues 23-193 of SEQ ID No. 17, and B represents at least one amino acid residue of the amino acid sequence designated by residues 194-250 of SEQ ID No. 17. In certain preferred embodiments, A and B together represent a contiguous polypeptide sequence designated sequence, A represents at least 25, 50, 75, 100, 125 or 150 amino acids of the designated sequence, and B represents at least 5, 10, or 20 amino acid residues of the amino acid sequence designated by corresponding entry in the sequence listing, and A and B together preferably represent a contiguous sequence corresponding to the sequence listing entry. Similar fragments from other hedgehog also

contemplated, e.g., fragments which correspond to the preferred fragments from the sequence listing entries which are enumerated above.

III. Production of Recombinant Polypeptides

Isolated hedgehog polypeptides described herein can be produced by any suitable method known in the art. Such methods range from direct protein synthetic methods to constructing a DNA sequence encoding isolated polypeptide sequences and expressing those sequences in a suitable transformed host.

In one embodiment of a recombinant method, a DNA sequence is constructed by isolating or synthesizing a DNA sequence encoding a wild type protein of interest. Optionally, the sequence may be mutagenized by site-specific mutagenesis to provide functional analogs thereof. See, e.g., United States Patent 4,588,585. Another method of constructing a DNA sequence encoding a polypeptide of interest would be by chemical synthesis using an oligonucleotide synthesizer. Such oligonucleotides may be preferably designed based on the amino acid sequence of the desired polypeptide, and preferably selecting those codons that are favored in the host cell in which the recombinant polypeptide of interest will be produced.

Standard methods may be applied to synthesize an isolated polynucleotide sequence encoding an isolated polypeptide of interest. For example, a complete amino acid sequence may be used to construct a back-translated gene. See Maniatis et al., supra. Further, a DNA oligomer containing a nucleotide sequence coding for the particular isolated polypeptide may be synthesized. For example, several small oligonucleotides coding for portions of the desired polypeptide may be synthesized and then ligated. The individual oligonucleotides typically contain 5' or 3' overhangs for complementary assembly.

Once assembled (by synthesis, site-directed mutagenesis, or by another method), the mutant DNA sequences encoding a particular isolated polypeptide of interest will be inserted into an expression vector and operatively linked to an expression control sequence appropriate for expression of the protein in a desired host. Proper assembly

enzyme, the size of the polypeptide, how easily the polypeptide is proteolytically degraded, and the like. The choice of a vector and insertion site for a given DNA is determined by a balance of these factors.

To provide for adequate transcription of the recombinant constructs of the invention, a suitable promoter/enhancer sequence may preferably be incorporated into the recombinant vector, provided that the promoter/expression control sequence is capable of driving transcription of a nucleotide sequence encoding a hedgehog protein. Any of a wide variety of expression control sequences may be used in these vectors. Such useful expression control sequences include the expression control sequences associated with structural genes of the foregoing expression vectors. Examples of useful expression control sequences include, for example, the-early and late promoters of SV40 or adenovirus, the lac system, the trp system, the TAC or TRC system, the major operator and promoter regions of phage lambda, for example pL, the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast alpha-mating system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells and their viruses, and various combinations thereof.

Promoters which may be used to control the expression of immunoglobulin-based fusion protein include, but are not limited to, the SV40 early promoter region (Benoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:144-1445), the regulatory sequences of the metallothionine gene (Brinster et al., 1982, Nature 296:39-42); plant expression vectors comprising the nopaline synthetase promoter region (Herrera-Estrella et al., Nature 303:209-213) or the cauliflower mosaic virus 35S RNA promoter (Gardner, et al., 1981, Nucl. Acids Res. 9:2871), and the promoter for the photosynthetic enzyme ribulose biphosphate carboxylase (Herrera-Estrella et al., 1984, Nature 310:115-120); promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phophatase promoter, and

the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene enhancers or promoters which are active in pancreatic cells (Hanahan, 1985, Nature 315:115-122); immunoglobulin gene enhancers or promoters which are active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444); the cytomegalovirus early promoter and enhancer regions (Boshart et al., 1985, Cell 41:521-530); mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495); albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276); alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58); alphantitrypsin gene control region which is active in the liver (Kelsey et al, 1987, Genes and Devel. 1:161-171); -globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94; myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286); and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

Any suitable host may be used to produce in quantity the isolated hedgehog polypeptides described herein, including bacteria, fungi (including yeasts), plants, insects, mammals, or other appropriate animal cells or cell lines, as well as transgenic animals or plants. More particularly, these hosts may include well known eukaryotic and prokaryotic hosts, such as strains of E. coli, Pseudomonas, Bacillus, Streptomyces, fungi, yeast (e.g., Hansenula), insect cells such as Spodoptera frugiperda (SF9), and High Five TM , animal cells such as Chinese hamster ovary (CHO), mouse cells such as

NS/O cells, African green monkey cells, COS 1, COS 7, BSC 1, BSC 40, and BMT 10, and human cells, as well as plant cells.

It should be understood that not all vectors and expression control sequences will function equally well to express a given isolated polypeptide. Neither will all hosts function equally well with the same expression system. However, one of skill in the art may make a selection among these vectors, expression control systems and hosts without undue experimentation. For example, to produce isolated polypeptide of interest in large-scale animal culture, the copy number of the expression vector must be controlled. Amplifiable vectors are well known in the art. See, for example, Kaufman and Sharp, (1982) Mol. Cell. Biol., 2, 1304-1319 and U.S. Patents 4,470,461 and 5,122,464.

Such operative linking of a DNA sequence to an expression control sequence includes the provision of a translation start signal in the correct reading frame upstream of the DNA sequence. If the particular DNA sequence being expressed does not begin with a methionine, the start signal will result in an additional amino acid (methionine) being located at the N-terminus of the product. If a hydrophobic moiety is to be linked to the N-terminal methionyl-containing protein, the protein may be employed directly in the compositions of the invention. Neverthless, since the preferred N-terminal end of the protein is to consist of a cysteine (or functional equivalent) the methionine must be removed before use. Methods are available in the art to remove such N-terminal methionines from polypeptides expressed with them. For example, certain hosts and fermentation conditions permit removal of substantially all of the N-terminal methionine in vivo. Other hosts require in vitro removal of the N-terminal methionine. Such in vitro and in vivo methods are well known in the art.

Successful incorporation of these polynucleotide constructs into a given expression vector may be identified by three general approaches: (a) DNA-DNA hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of the hedgehog gene inserted in an expression vector can be detected by DNA-DNA hybridization using probes comprising sequences that are homologous to the inserted fusion protein gene. In the

second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics such as G418, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign genes in the vector. For example, if the polynucleotide is inserted so as to interrupt a marker gene sequence of the vector, recombinants containing the insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the foreign gene product expressed by the recombinant vector. Such assays can be based, for example, on the physical or functional properties of the gene product in bioassay systems.

Recombinant nucleic acid molecules which encode chimeric hedgehog therapeutics may be obtained by any method known in the art (Maniatis et al., 1982, Molecular Cloning; A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) or obtained from publicly available clones. Methods for the preparation of genes which encode the heavy or light chain constant regions of immunoglobulins are taught, for example, by Robinson, R. et al., PCT Application, Publication No. W087-02671. The cDNA sequence encoding the hedgehog molecule or fragment may be directly joined to the cDNA encoding the heavy Ig contant regions or may be joined via a linker sequence. In further embodiments of the invention, a recombinant vector system may be created to accommodate sequences encoding hedgehog in the correct reading frame with a synthetic hinge region. Additionally, it may be desirable to include, as part of the recombinant vector system, nucleic acids corresponding to the 3' flanking region of an immunoglobulin gene including RNA cleavage/polyadenylation sites and downstream sequences. Furthermore, it may be desirable to engineer a signal sequence upstream of the immunoglobulin fusion protein-encoding sequences to facilitate the secretion of the fused molecule from a cell transformed with the recombinant vector.

The proteins produced by a transformed host can be purified according to any suitable method. Such standard methods include chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for protein purification. For immunoaffinity

chromatography (See Example), a protein such as Sonic hedgehog may be isolated by binding it to an affinity column comprising of antibodies that were raised against Sonic hedgehog, or a related protein and were affixed to a stationary support. For example, the hedgehog proteins and fragments may be purified by passing a solution thereof through a column having an hedgehog receptor immobilized thereon (see U.S.Pat. No. 4,725,669). The bound hedgehog molecule may then be eluted by treatment with a chaotropic salt or by elution with aqueous acetic acid. Specific immunoglobulin fusion proteins may be purified by passing a solution containing the fusion protein through a column which contains immobilized protein A or protein G which selectively binds the Fc portion of the fusion protein. See, for example, Reis, K. J., et al., J. Immunol. 132:3098-3102 (1984); PCT Application, Publication No. W087/00329.

Alternatively hedgehog proteins and chimeric molecules may be purified on anti-hedgehog antibody columns, or on anti-immunoglobulin antibody columns to give a substantially pure protein. By the term "substantially pure" is intended that the protein is free of the impurities that are naturally associated therewith. Substantial purity may be evidenced by a single band by electrophoresis. Alternatively, affinity tags such as hexahistidine, maltose binding domain, influenza coat sequence, and glutathione-S-transferase can be attached to the protein to allow easy purification by passage over an appropriate affinity column. Isolated proteins can also be characterized physically using such techniques as proteolysis, nuclear magnetic resonance, and X-ray crystallography.

An example of a useful hedgehog/Ig chimeric protein of this invention is that protein encoded by the nucleotide sequence of SEQ ID NOS: 31-34, which are secreted into the cell culture by eukaryotic cells containing the expression plasmids pUB55, pUB 114, pUB 115 and pUB 116, respectively (See Examples). These proteins consist of the mature human hedgehog fused to a portion of the hinge region and the CH2 and CH3 constant domains of murine or human Ig. Proteins of this group contains a sufficient portion of the immunoglobulin to be recognized by the Fc binding protein, Protein A.

A. Production of Fragments and Analogs

Fragments of an isolated protein (e.g., fragments of SEQ ID NOS: 10-18 or 23-26) can also be produced efficiently by recombinant methods, by proteolytic digestion, or by chemical synthesis using methods known to those of skill in the art. In recombinant methods, internal or terminal fragments of a polypeptide can be generated by removing one or more nucleotides from one end (for a terminal fragment) or both ends (for an internal fragment) of a DNA sequence which encodes for the isolated hedgehog polypeptide. Expression of the mutagenized DNA produces polypeptide fragments. Digestion with "end nibbling" endonucleases can also generate DNAs which encode an array of fragments. DNAs which encode fragments of a protein can also be generated by random shearing, restriction digestion, or a combination of both. Protein fragments can be generated directly from intact proteins. Peptides can be cleaved specifically by proteolytic enzymes, including, but not limited to plasmin, thrombin, trypsin, chymotrypsin, or pepsin. Each of these enzymes is specific for the type of peptide bond it attacks. Trypsin catalyzes the hydrolysis of peptide bonds in which the carbonyl group is from a basic amino acid, usually arginine or lysine. Pepsin and chymotrypsin catalyse the hydrolysis of peptide bonds from aromatic amino acids, such as tryptophan, tyrosine, and phenylalanine. Alternative sets of cleaved protein fragments are generated by preventing cleavage at a site which is susceptible to a proteolytic enzyme. For instance, reaction of the E-amino acid group of lysine with ethyltrifluorothioacetate in mildly basic solution yields blocked amino acid residues whose adjacent peptide bond is no longer susceptible to hydrolysis by trypsin. Proteins can be modified to create peptide linkages that are susceptible to proteolytic enzymes. For instance, alkylation of cysteine residues with (3-haloethylamines yields peptide linkages that are hydrolyzed by trypsin (Lindley, (1956) Nature 178, 647). In addition, chemical reagents that cleave peptide chains at specific residues can be used. For example, cyanogen bromide cleaves peptides at methionine residues (Gross and Witkip, (1961) J. Am. Chem. Soc. 83, 1510). Thus, by treating proteins with various combinations of modifiers, proteolytic enzymes and/or chemical reagents, the proteins

may be divided into fragments of a desired length with no overlap of the fragments, or divided into overlapping fragments of a desired length.

Fragments can also be synthesized chemically using techniques known in the art such as the Merrifield solid phase F moc or t-Boc chemistry. Merrifield, Recent Progress in Hormone Research 23: 451 (1967).

Examples of prior art methods which allow production and testing of fragments and analogs are discussed below. These, or analogous methods may be used to make and screen fragments and analogs of an isolated polypeptide (e.g., hedgehog) which can be shown to have biological activity. An exemplary method to test whether fragments and analogs of hedgehog have biological activity is found in Example -

B. Production of Altered DNA and Peptide Sequences: Random Methods

Amino acid sequence variants of a protein can be prepared by random mutagenesis of DNA which encodes the protein or a particular portion thereof. Useful methods include PCR mutagenesis and saturation mutagenesis. A library of random amino acid sequence variants can also be generated by the synthesis of a set of degenerate oligonucleotide sequences. Methods of generating amino acid sequence variants of a given protein using altered DNA and peptides are well-known in the art. The following examples of such methods are not intended to limit the scope of the present invention, but merely serve to illustrate representative techniques. Persons having ordinary skill in the art will recognize that other methods are also useful in this regard.

<u>PCR Mutagenesis</u>: See, for example Leung et al., (1989) Technique 1, 11-15.

<u>Saturation Mutagenesis</u>: One method is described generally in Mayers et al., (1989) Science 229, 242.

<u>Degenerate Oligonucleotide Mutagenesis</u>: See for example Harang, S.A., (1983) Tetrahedron 39, 3; Itakura et al., (1984) Ann. Rev. Biochem. 53, 323 and Itakura et al., Recombinant DNA, Proc. 3rd Cleveland Symposium on Macromolecules, pp. 273-289 (A.G. Walton, ed.), Elsevier, Amsterdam, 1981.

C. Production of Altered DNA and Peptide Sequences: Directed Methods

Non-random, or directed, mutagenesis provides specific sequences or mutations in specific portions of a polynucleotide sequence that encodes an isolated polypeptide, to provide variants which include deletions, insertions, or substitutions of residues of the known amino acid sequence of the isolated polypeptide. The mutation sites may be modified individually or in series, for instance by: (1) substituting first with conserved amino acids and then with more radical choices depending on the results achieved; (2) deleting the target residue; or (3) inserting residues of the same or a different class adjacent to the located site, or combinations of options 1-3.

Clearly, such site-directed methods are one way in which an N-terminal cysteine (or a functional equivalent) can be introduced into a given polypeptide sequence to provide the attachment site for a hydrophobic moiety.

Alanine scanning Mutagenesis: See Cunningham and Wells, (1989) Science 244, 1081-1085).

Oligonucleotide-Mediated Mutagenesis: See, for example, Adelman et al., (1983) DNA 2, 183.

Cassette Mutagenesis: See Wells et al., (1985) Gene 34, 315.

Combinatorial Mutagenesis: See, for example, Ladner et al., WO 88/06630

Indeed, it is plain from the combinatorial mutagenesis art that large scale mutagenesis of hedgehog proteins, without any preconceived ideas of which residues were critical to the biological function, and generate wide arrays of variants having equivalent biological activity. Indeed, it is the ability of combinatorial techniques to screen billions of different variants by high throughout analysis that removes any requirement of a priori understanding or knowledge of critical residues.

D. Other Variants of Isolated Polypeptides

Included in the invention are isolated molecules that are: allelic variants, natural mutants, induced mutants, and proteins encoded by DNA that hybridizes under high or low stringency conditions to a nucleic acid which encodes a polypeptide such as the N-

terminal fragment of Sonic hedgehog (SEQ ID NO: 23) and polypeptides bound specifically by antisera to hedgehog peptides, especially by antisera to an active site or binding site of hedgehog. All variants described herein are expected to: (i) retain the biological function of the original protein and (ii) retain the ability to link to form a chimeric molecule with a non-hedgehog moiety.

The methods of the invention also feature uses of fragments, preferably biologically active fragments, or analogs of an isolated peptide such as hedgehog. Specifically, a biologically active fragment or analog is one having any in vivo or in vitro activity which is characteristic of the peptide shown in SEQ ID NOS: 10-18 or 23-26 or of other naturally occurring isolated hedgehog. Most preferably, the hydrophobically-modified fragment or analog has at least 10%, preferably 40% or greater, or most preferably at least 90% of the activity of Sonic hedgehog in any in vivo or in vitro assay.

Analogs can differ from naturally occurring isolated protein in amino acid sequence or in ways that do not involve sequence, or both. The most preferred polypeptides of the invention have preferred non-sequence modifications that include in vivo or in vitro chemical derivatization (e.g., of their N-terminal end). *Hedgehog* polypeptides may also be chemically modified to create *hedgehog* derivatives by forming covalent or aggregate conjugates with other chemical moieties, such as glycosyl groups, cholesterol, isoprenoids, lipids, phosphate, acetyl groups and the like. Covalent derivatives of *hedgehog* proteins can be prepared by linking the chemical moieties to functional groups on amino acid sidechains of the protein or at the N-terminus or at the C-terminus of the polypeptide.

For instance, *hedgehog* proteins can be generated to include a moiety, other than sequence naturally associated with the protein, that binds a component of the extracellular matrix and enhances localization of the analog to cell surfaces. For example, sequences derived from the fibronectin "type-III repeat", such as a tetrapeptide sequence R-G-D-S (Pierschbacher et al. (1984) *Nature* 309:30-3; and Kornblihtt et al. (1985) *EMBO* 4:1755-9) can be added to the *hedgehog* polypeptide to support attachment of the chimeric molecule to a cell through binding ECM components

(Ruoslahti et al. (1987) *Science* 238:491-497; Pierschbacheret al. (1987) *J. Biol. Chem.* 262:17294-8.; Hynes (1987) *Cell* 48:549-54; and Hynes (1992) *Cell* 69:11-25).

Other analogs include a protein such as Sonic hedgehog or its biologically active fragments whose sequences differ from the wild type consensus sequence (e.g., SEQ ID NO: 26) by one or more conservative amino acid substitutions or by one or more non conservative amino acid substitutions, or by deletions or insertions which do not abolish the isolated protein's biological activity. Conservative substitutions typically include the substitution of one amino acid for another with similar characteristics such as substitutions within the following groups: valine, alanine and glycine; leucine and isoleucine; aspartic acid and glutamic acid; asparagine and glutamine; serine and threonine; lysine and arginine; and phenylalanine and tyrosine. The non-polar hydrophobic amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine, and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Other conservative substitutions can be readily known by workers of ordinary skill. For example, for the amino acid alanine, a conservative substitution can be taken from any one of D-alanine, glycine, beta-alanine, L-cysteine, and D-cysteine. For lysine, a replacement can be any one of D-lysine, arginine, D-arginine, homo-arginine, methionine, D-methionine, ornithine, or D-ornithine.

Other analogs used within the methods of the invention are those with modifications which increase peptide stability. Such analogs may contain, for example, one or more non-peptide bonds (which replace the peptide bonds) in the peptide sequence. Also included are: analogs that include residues other than naturally occurring L-amino acids, such as D-amino acids or non-naturally occurring or synthetic amino acids such as beta or gamma amino acids and cyclic analogs. Incorporation of D- instead of L-amino acids into the isolated hedgehog polypeptide may increase its resistance to proteases. See, U.S. Patent 5,219,990 supra. The term "fragment", as applied to an isolated hedgehog analog, can be as small as a single amino acid provided that it retains

biological activity. It may be at least about 20 residues, more typically at least about 40 residues, preferably at least about 60 residues in length. Fragments can be generated by methods known to those skilled in the art. The ability of a candidate fragment to exhibit isolated hedgehog biological activity can be also assessed by methods known to those skilled in the art as described herein.

IV. Antagonists of Hedgehog Activity

A preferred antagonist has at least the following properties: (i) the isolated protein binds the receptor patched-1 with an affinity that may be less than, but is preferably at least the same as, the binding of mature hedgehog protein to patched-1; and (ii) the isolated protein blocks alkaline phosphatase (AP) induction by mature hedgehog protein when tested in an in vitro CH310T1/2 cell-based AP induction assay. Antagonists of the invention may also have the additional properties of being (iii) unable to induce ptc-1 and gli-1 expression.

Persons having ordinary skill in the art can easily test any putative hedgehog antagonist for these properties. In particular, the mouse embryonic fibroblast line C3H10T1/2 is a mesenchymal stem cell line that is hedgehog responsive. Hedgehog treatment of the cells causes an upregulation of gli-1 and patched-1, (known indicators of hedgehog dependent signaling) and also causes induction of alkaline phosphatase activity, an indicator that the cells have differentiated down the chondrocyte/ bone osteoblast lineage. Several hedgehog variants are unable to elicit a hedgehog-dependent response on C3H10T1/2 cells, but they competed with mature hedgehog for function and therefore serve as functional antagonists. The synthesis and use of such hedgehog antagonist moieties are briefly described below.

A. N-Modified Hedgehog Polypeptides as Antagonists

Certain hedgehog variants that contain N-terminal modifications can block hedgehog function because they lack the ability to elicit a hedgehog-dependent response but retain the ability to bind to hedgehog receptor, patched-1. The critical primary amino acid sequence that defines whether a hedgehog polypeptide (i.e., a Sonic, Indian or

Desert hedgehog) is a functional hedgehog antagonist is the N-terminal cysteine residue which corresponds to Cys-1 of the mature hedgehog. So long as the hedgehog polypeptide either lacks this N-terminal cysteine completely or contains this N-terminal cysteine in a modified form (e.g. chemically modified or included as part of an N-terminal extension moiety), the resulting polypeptide can act as a functional hedgehog antagonist. In this regard, the fact that an N-terminal cysteine "corresponds to Cys-1" means: (a) the N-terminal cysteine is the Cys-1 of mature Sonic, Indian or Desert hedgehog; or (b) the N-terminal cysteine occupies the same position as Cys-1 of mature Sonic, Indian or Desert hedgehog. Provided that, for example, a Sonic hedgehog has an N-terminal cysteine corresponding to Cys-1 that is altered or otherwise modified as described herein, it can antagonize the action of any other member of the hedgehog family. Therefore, persons having ordinary skill in the art will understand that it is possible for an Indian hedgehog protein to antagonize the activity of Sonic, Desert or Indian hedgehogs.

Examples of these antagonists with N-terminal modifications are included below and one skilled in the art can alter the disclosed structure of the antagonist, e.g., by producing fragments or analogs, and test the newly produced structures for antagonist activity. These examples in no way limit the structure of any related hedgehog antagonists, but are merely provided for further description. These, or analogous methods, can be used to make and screen fragments and analogs of a antagonist polypeptides. There are several variants that are able to function as antagonists.

1. N-terminal extensions

Antagonist polypeptides of the invention may include a hedgehog polypeptide sequence in which the N-terminal cysteine is linked to an N-terminal extension moiety. The isolated antagonist polypeptide can therefore be, as but one example, a recombinant fusion protein having: (a) a first N-terminal polypeptide portion that can be 5' to the hedgehog polypeptide itself, and that contains at least one element (e.g., an amino acid residue) that may be unrelated to hedgehog, linked to (b) an N-terminal cysteine corresponding to Cys-1 of Sonic hedgehog that is part of a hedgehog antagonist of the invention, or a portion of hedgehog antagonist. This N-terminal extension moiety (e.g.,

the first N-terminal polypeptide portion) can be a histidine tag, a maltose binding protein, glutathione-S-transferase, a DNA binding domain, or a polymerase activating domain. The functional antagonist may include an N-terminal extension moiety that contains an element which replaces the Cys-1 of mature hedgehog or an N-terminal cysteine that corresponds to Cys-1 of a mature Sonic hedgehog.

2. N-terminal deletions

Another variation of a functional antagonist is a hedgehog protein that is missing no greater than about 12 amino acids beginning from that N-terminal cysteine corresponding to Cys-1 of a mature hedgehog. Deletions in more than the about the first 12 contiguous amino acid residues do not generate functional antagonists. Preferably, deletions of about 10 contiguous amino acids will provide suitable functional antagonists. One can, however, remove fewer than 10 contiguous residues and still maintain antagonist function. Moreover, one can delete various combinations of non-contiguous residues provided that there are at least about 3 deleted residues in total.

These structures highlight the importance of the N-terminus of hedgehog proteins for function and indeed, underscore the need to conjugate a hedgehog protein at a site other than the N-terminal cysteine. All of the N-terminal deletion variants were indistinguishable from mature Sonic hedgehog (Shh) in their ability to bind patched-1, but were inactive in the in vitro C3H10T1/2 AP induction assay. All these N-terminal variants are unable to promote hedgehog-dependent signaling.

3. N-terminal mutations

Yet another functional antagonist has a mutation of the N-terminal cysteine to another amino acid residue. Any non-hydrophobic amino acid residue may be acceptable and persons having ordinary skill in the art following the teachings described herein will be able to perform the mutations and test the effects of such mutations. One example is Shh in which the N-terminal cysteine is replaced with a serine residue. This mutated form is indistinguishable from mature Shh in its ability to bind patched-1, but it blocks AP induction by mature Shh when tested for function in the C3H10T1/2 AP induction assay. Replacements with aspartic acid, alanine and histidine have also shown to serve as antagonists.

4. N-terminal cysteine modifications

Because the primary amino acid sequence of hedgehog contains the Cys-1 that is important for biological activity, certain other modifications will result in inactive antagonist variants of hedgehog protein. Another antagonist is an isolated functional antagonist of a hedgehog polypeptide, comprising a hedgehog polypeptide containing an N-terminal cysteine that corresponds to Cys-1 of a mature Sonic hedgehog, except that the cysteine is in a modified form. Antagonist polypeptides of hedgehog may have non-sequence modifications that include in vivo or in vitro chemical derivatization of their N-terminal cysteine, as well as possible changes in acetylation, methylation, phosphorylation, amidation, or carboxylation. As an example, the functional antagonist can have an N-terminal cysteine in an oxidized form. Thus, a functional antagonist can have an N-terminal cysteine that is effectively modified by including it as part of an N-terminal extension moiety.

The functional antagonist polypeptides can include amino acid sequences that are at least 60% homologous to a hedgehog protein. The antagonist must exhibit at least the following functional antagonist properties: (i) the isolated protein binds the receptor patched-1 with an affinity that may be less than, but is preferably at least the same as, the binding of mature hedgehog protein to patched-1; and (ii) the isolated protein blocks alkaline phosphatase (AP) induction by mature hedgehog protein when tested in an in vitro CH310T1/2 cell-based AP induction assay.

Antagonists useful in the present invention also include those which arise as a result of the existence of multiple genes, alternative transcription events, alternative RNA splicing events, and alternative translational and posttranslational events. The polypeptide can be made entirely by synthetic means or can be expressed in systems, e.g., cultured cells, which result in substantially the same posttranslational modifications present when the protein is expressed in a native cell, or in systems which result in the omission of posttranslational modifications present when expressed in a native cell.

In a preferred embodiment, isolated antagonist is a polypeptide with one or more of the following characteristics:

(i) it has at least 60, more preferably 90 and most preferably 95% sequence identity with amino acids of SEQ ID NOS: 10-18 and 23-26;

(ii) it either has a modified N-terminal cysteine or lacks an N-terminal cysteine or has an N-terminal cysteine in a position different from the N-terminal cysteine corresponding to Cys-1 of the hedgehog;

(iii) it blocks alkaline phosphatase induction by mature hedgehog in CH310T1/2 cells;

(iv) it binds or interacts with its receptor patched-1 with an affinity that may be less than, but is preferably at least the same as, the binding of mature hedgehog protein to patched-1;

(v) it is unable to induce ptc-1 and gli-1 expression in vitro in CH310T1/2 cells; or

(vi) it is unable to induce AP in CH310T1/2 assays.

B. Antibody Homologs as Antagonists

In other embodiments, the antagonists used in the method of the invention to bind to, including block or coat, cell-surface hedgehog (such as vertebrate Sonic, Indian or Desert) and/or cell surface ligand for said hedgehog proteins (such as patched) is an anti-hedgehog and/or anti patched monoclonal antibody or antibody homolog, as defined previously. Preferred antibodies and homologs for treatment, in particular for human treatment, include human antibody homologs, humanized antibody homologs, chimeric antibody homologs, Fab, Fab', F(ab')2 and F(v) antibody fragments, and monomers or dimers of antibody heavy or light chains or mixtures thereof. Monoclonal antibodies against VLA-4 are a preferred binding agent in the method of the invention.

The technology for producing monoclonal antibodies is well known. The preferred antibody homologs contemplated herein can be expressed from intact or truncated genomic or cDNA or from synthetic DNAs in prokaryotic or eukaryotic host cells. The dimeric proteins can be isolated from the culture media and/or refolded and dimerized in vitro to form biologically active compositions. Heterodimers can be formed in vitro by combining separate, distinct polypeptide chains. Alternatively,

heterodimers can be formed in a single cell by co-expressing nucleic acids encoding separate, distinct polypeptide chains. See, for example, W093/09229, or U.S. Pat. No. 5,411,941, for several exemplary recombinant heterodimer protein production protocols. Currently preferred host cells include, without limitation, prokaryotes including E. coli, or eukaryotes including yeast, Saccharomyces, insect cells, or mammalian cells, such as CHO, COS or BSC cells. One of ordinary skill in the art will appreciate that other host cells can be used to advantage. For example, anti-hedgehog antibodies may be identified by immunoprecipitation of 1251-labeled cell lysates from hedgehog -expressing cells. Anti-hedgehog antibodies may also be identified by flow cytometry, e.g., by measuring fluorescent staining of cells incubated with an antibody believed to recognize hedgehog protein. The lymphocytes used in the production of hybridoma cells typically are isolated from immunized mammals whose sera have already tested positive for the presence of anti-hedgehog antibodies using such screening assays.

Typically, the immortal cell line (e.g., a myeloma cell line) is derived from the same mammalian species as the lymphocytes. Preferred immortal cell lines are mouse myeloma cell lines that are sensitive to culture medium containing hypoxanthine, arninopterin and thymidine ("HAT medium"). Typically, HAT-sensitive mouse myeloma cells are fused to mouse splenocytes using 1500 molecular weight polyethylene glycol ("PEG 1500"). Hybridoma cells resulting from the fusion are then selected using HAT medium, which kills unfused and unproductively fused myeloma cells (unfused splenocytes die after several days because they are not transformed). Hybridomas producing a desired antibody are detected by screening the hybridoma culture supernatants. For example, hybridomas prepared to produce anti-hedgehog or patched antibodies may be screened by testing the hybridoma culture supernatant for secreted antibodies having the ability to bind to a recombinant hedgehog or patched expressing cell line.

To produce antibody homologs that are intact immunoglobulins, hybridoma cells that tested positive in such screening assays were cultured in a nutrient medium under conditions and for a time sufficient to allow the hybridoma cells to secrete the monoclonal antibodies into the culture medium. Tissue culture techniques and culture

media suitable for hybridoma cells are well known. The conditioned hybridoma culture supernatant may be collected and the anti-hedgehog or patched antibodies optionally further purified by well-known methods.

Alternatively, the desired antibody may be produced by injecting the hybridoma cells into the peritoneal cavity of an unimmunized mouse. The hybridoma cells proliferate in the peritoneal cavity, secreting the antibody which accumulates as ascites fluid. The antibody may be harvested by withdrawing the ascites fluid from the peritoneal cavity with a syringe. Several anti-hedgehog or patched monoclonal antibodies have been previously described. These anti-hedgehog or patched monoclonal antibodies and others will be useful in the methods of treatment according to the present invention.

Fully human monoclonal antibody homologs against hedgehog or patched are another preferred binding agent which may block or coat hedgehog ligands in the method of the invention. In their intact form these may be prepared using in vitro-primed human splenocytes, as described by Boerner et al., 1991, J. Immunol., 147, 86-95. Alternatively, they may be prepared by repertoire cloning as described by Persson et al., 1991, Proc. Nat. Acad. Sci. USA, 88: 2432-2436 or by Huang and Stollar, 1991, J. Immunol. Methods 141, 227-236. U.S. Patent 5,798,230 (Aug. 25, 1998, "Process for the preparation of human monoclonal antibodies and their use") who describe preparation of human monoclonal antibodies from human B cells. According to this process, human antibody-producing B cells are immortalized by infection with an Epstein-Barr virus, or a derivative thereof, that expresses Epstein-Barr virus nuclear antigen 2 (EBNA2). EBNA2 function, which is required for immortalization, is subsequently shut off, which results in an increase in antibody production.

In yet another method for producing fully human antibodies, United States Patent 5,789,650 (Aug. 4, 1998, "Transgenic non-human animals for producing heterologous antibodies") describes transgenic non-human animals capable of producing heterologous antibodies and transgenic non-human animals having inactivated endogenous immunoglobulin genes. Endogenous immunoglobulin genes are suppressed by antisense polynucleotides and/or by antiserum directed against endogenous

immunoglobulins. Heterologous antibodies are encoded by immunoglobulin genes not normally found in the genome of that species of non-human animal. One or more transgenes containing sequences of unrearranged heterologous human immunoglobulin heavy chains are introduced into a non-human animal thereby forming a transgenic animal capable of functionally rearranging transgenic immunoglobulin sequences and producing a repertoire of antibodies of various isotypes encoded by human immunoglobulin genes. Such heterologous human antibodies are produced in B-cells which are thereafter immortalized, e.g., by fusing with an immortalizing cell line such as a myeloma or by manipulating such B-cells by other techniques to perpetuate a cell line capable of producing a monoclonal heterologous, fully human antibody homolog.

Large nonimmunized human phage display libraries may also be used to isolate high affinity antibodies that can be developed as human therapeutics using standard phage technology (Vaughan et al, 1996).

Yet another preferred binding agent which may block or coat hedgehog ligands in the method of the invention is a humanized recombinant antibody homolog having anti-hedgehog or patched specificity. Following the early methods for the preparation of true "chimeric antibodies" (where the entire constant and entire variable regions are derived from different sources), a new approach was described in EP 0239400 (Winter et al.) whereby antibodies are altered by substitution (within a given variable region) of their complementarity determining regions (CDRs) for one species with those from another. This process may be used, for example, to substitute the CDRs from human heavy and light chain Ig variable region domains with alternative CDRs from murine variable region domains. These altered Ig variable regions may subsequently be combined with human Ig constant regions to create antibodies which are totally human in composition except for the substituted murine CDRs. Such CDR-substituted antibodies would be predicted to be less likely to elicit an immune response in humans compared to true chimeric antibodies because the CDR-substituted antibodies contain considerably less non-human components. The process for humanizing monoclonal antibodies via CDR "grafting" has been termed "reshaping". (Riechmann et al., 1988, Nature 332, 323-327; Verhoeyen et al., 1988, Science 239, 1534-1536).

Typically, complementarity determining regions (CDRs) of a murine antibody are transplanted onto the corresponding regions in a human antibody, since it is the CDRs (three in antibody heavy chains, three in light chains) that are the regions of the mouse antibody which bind to a specific antigen. Transplantation of CDRs is achieved by genetic engineering whereby CDR DNA sequences are determined by cloning of murine heavy and light chain variable (V) region gene segments, and are then transferred to corresponding human V regions by site directed mutagenesis. In the final stage of the process, human constant region gene segments of the desired isotype (usually gamma I for CH and kappa for CL) are added and the humanized heavy and light chain genes are co-expressed in mammalian cells to produce soluble humanized antibody.

The transfer of these CDRs to a human antibody confers on this antibody the antigen binding properties of the original murine antibody. The six CDRs in the murine antibody are mounted structurally on a V region "framework" region. The reason that CDR-grafting is successful is that framework regions between mouse and human antibodies may have very similar 3-D structures with similar points of attachment for CDRS, such that CDRs can be interchanged. Such humanized antibody homologs may be prepared, as exemplified in Jones et al., 1986, Nature 321, 522-525; Riechmann, 1988, Nature 332, 323-327; Queen et al., 1989, Proc. Nat. Acad. Sci. USA 86, 10029; and Orlandi et al., 1989, Proc. Nat. Acad. Sci. USA 86, 3833.

Nonetheless, certain amino acids within framework regions are thought to interact with CDRs and to influence overall antigen binding affinity. The direct transfer of CDRs from a murine antibody to produce a recombinant humanized antibody without any modifications of the human V region frameworks often results in a partial or complete loss of binding affinity. In a number of cases, it appears to be critical to alter residues in the framework regions of the acceptor antibody in order to obtain binding activity.

Queen et al., 1989 (supra) and WO 90/07861 (Protein Design Labs) have described the preparation of a humanized antibody that contains modified residues in the framework regions of the acceptor antibody by combining the CDRs of a murine MAb

(anti-Tac) with human immunoglobulin framework and constant regions. They have demonstrated one solution to the problem of the loss of binding affinity that often results from direct CDR transfer without any modifications of the human V region framework residues; their solution involves two key steps. First, the human V framework regions are chosen by computer analysts for optimal protein sequence homology to the V region framework of the original murine antibody, in this case, the anti-Tac MAb. In the second step, the tertiary structure of the murine V region is modelled by computer in order to visualize framework amino acid residues which are likely to interact with the murine CDRs and these murine amino acid residues are then superimposed on the homologous human framework. See also U.S. Patents 5,693,762; 5,693,761; 5,585,089; and 5,530,101 (Protein Design Labs).

One may use a different approach (Tempest et al.,1991, Biotechnology 9, 266-271) and utilize, as standard, the V region frameworks derived from NEWM and REI heavy and light chains respectively for CDR-grafting without radical introduction of mouse residues. An advantage of using the Tempest et al., approach to construct NEWM and REI based humanized antibodies is that the 3dimensional structures of NEWM and REI variable regions are known from x-ray crystallography and thus specific interactions between CDRs and V region framework residues can be modeled.

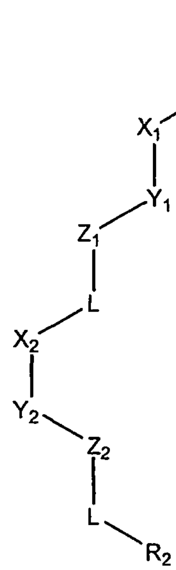
Regardless of the approach taken, the examples of the initial humanized antibody homologs prepared to date have shown that it is not a straightforward process. However, even acknowledging that such framework changes may be necessary, it is not possible to predict, on the basis of the available prior art, which, if any, framework residues will need to be altered to obtain functional humanized recombinant antibodies of the desired specificity. Results thus far indicate that changes necessary to preserve specificity and/or affinity are for the most part unique to a given antibody and cannot be predicted based on the humanization of a different antibody.

C. Small Organic Molecules as Antagonists

In other embodiments, a hedgehog antagonist may be a small organic molecule. Such a small organic molecule may antagonize hedgehog signal transduction via an

interaction with but not limited to *hedgehog, patched* (*ptc*), *gli*, and/or *smoothened*. It is, therefore, specifically contemplated that these small molecules which intefere with aspects of *hedgehog*, *ptc*, or *smoothened* signal transduction activity will likewise be capable of inhibiting angiogenesis (or other biological consequences) in normal cells and/or mutant cells. Thus, it is contemplated that in certain embodiments, these compounds may be useful for inhibiting *hedgehog* activity in normal cells. In other embodiments, these compounds may be useful for inhibitng *hedgehog* activity in abnormal cells. In preferred embodiments, the subject inhibitors are organic molecules having a molecular weight less than 2500 amu, more preferably less than 1500 amu, and even more preferably less than 750 amu, and are capable of antagonizing hedgehog signaling, preferably specifically in target cells.

For example, compounds useful in the subject methods include compounds may be represented by general forumla (I):



Formula I

wherein, as valence and stability permit,

R_1 and R_2, independently for each occurrence, represent H, lower alkyl, aryl (e.g., substituted or unsubstituted), aralkyl (e.g., substituted or unsubstituted, e.g., -

$(CH_2)_n$aryl), or heteroaryl (e.g., substituted or unsubstituted), or heteroaralkyl (e.g., substituted or unsubstituted, e.g., -$(CH_2)_n$heteroaralkyl-);

L, independently for each occurrence, is absent or represents -$(CH_2)_n$-alkyl, -alkenyl-, -alkynyl-, -$(CH_2)_n$alkenyl-, -$(CH_2)_n$alkynyl-, -$(CH_2)_nO(CH_2)_p$-, -$(CH_2)_nNR_2(CH_2)_p$-, -$(CH_2)_nS(CH_2)_p$-, -$(CH_2)_n$alkenyl$(CH_2)_p$-, -$(CH_2)_n$alkynyl$(CH_2)_p$-, -$O(CH_2)_n$-, -$NR_2(CH_2)_n$-, or -$S(CH_2)_n$-;

X_1 and X_2 can be selected, independently, from -N(R_8)-, -O-, -S-, -Se-, -N=N-, -ON=CH-, -(R_8)N-N(R_8)-, -ON(R_8)-, a heterocycle, or a direct bond between L and Y_1 or Y_2, respectively;

Y_1 and Y_2 can be selected, independently, from -C(=O)-, -C(=S)-, -S(O_2)-, -S(O)-, -C(=NCN)-, -P(=O)(OR_2)-, a heteroaromatic group, or a direct bond between X_1 and Z_1 or X_2 and Z_2, respectively;

Z_1 and Z_2 can be selected, independently, from -N(R_8)-, -O-, -S-, -Se-, -N=N-, -ON=CH-, -R_8N-NR_8-, -ONR_8-, a heterocycle, or a direct bond between Y_1 or Y_2, respectively, and L;

R_8, independently for each occurrence, represents H, lower alkyl, -$(CH_2)_n$aryl (e.g., substituted or unsubstituted), -$(CH_2)_n$heteroaryl (e.g., substituted or unsubstituted), or two R_8 taken together may form a 4- to 8-membered ring, e.g., with X_1 and Z_1 or X_2 and Z_1, which ring may include one or more carbonyls;

p represents, independently for each occurrence, an integer from 0 to 10, preferably from 0 to 3; and

n, individually for each occurence, represents an integer from 0 to 10, preferably from 0 to 5.

In certain embodiments, R_1 represents a substituted or unsubstituted heteroaryl group.

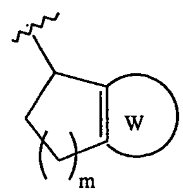
In certain embodiments, X_1 and X_2 can be selected from -N(R_8)-, -O-, -S-, a direct bond, and a heterocycle, Y_1 and Y_2 can be selected from -C(=O)-, -C(=S)-, and -

$S(O_2)$-, and Z_1 or Z_2 can be selected from -$N(R_8)$-, -O-, -S-, a direct bond, and a heterocycle.

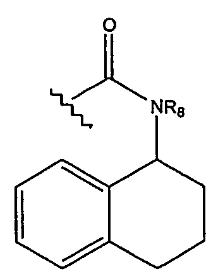
In certain related embodiments, X_1-Y_1-Z_1 or X_2-Y_2-Z_2 taken together represents a urea (N-C(O)-N) or an amide (N-C(O) or C(O)-N).

In certain embodiments, X_1 or X_2 represents a diazacarbocycle, such as a piperazine.

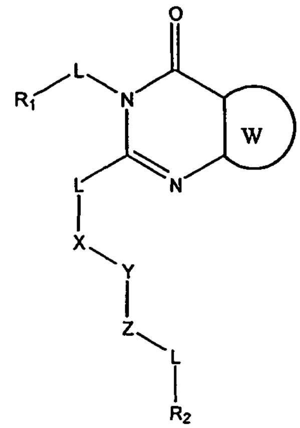
In certain embodiments, R_1 represents a fused cycloalkyl-aryl or cycloalkyl-heteroaryl system, for example:



wherein W is a substituted or unsubstituted aryl or heteroaryl ring fused to the cycloalkyl ring and m is an integer from 1-4 inclusive, e.g., from 1-3, or from 1-2. The fused system may be bound to L from any carbon of the fused system, including the position depicted above. In certain embodiments, R_1 may represent a tetrahydronaphthyl group, and preferably Y_1-X_1-L-R_1 taken together represent a tetrahydronaphthyl amide group, such as:



In embodiments wherein Y_1 and Z_1 are absent and X_1 comprises a pyrimidone, compounds useful in the present invention may be represented by general formula (II):



Formula II

wherein, as valence and stability permit,

R_1 and R_2, independently for each occurrence, represent H, lower alkyl, -$(CH_2)_n$aryl (e.g., substituted or unsubstituted), or -$(CH_2)_n$heteroaryl (e.g., substituted or unsubstituted);

L, independently for each occurrence, is absent or represents -$(CH_2)_n$-alkyl, -alkenyl-, -alkynyl-, -$(CH_2)_n$alkenyl-, -$(CH_2)_n$alkynyl-, -$(CH_2)_nO(CH_2)_p$-, -$(CH_2)_nNR_2(CH_2)_p$-, -$(CH_2)_nS(CH_2)_p$-, -$(CH_2)_n$alkenyl$(CH_2)_p$-, -$(CH_2)_n$alkynyl$(CH_2)_p$-, -$O(CH_2)_n$-, -$NR_2(CH_2)_n$-, or -$S(CH_2)_n$-;

X can be selected from -$N(R_8)$-, -O-, -S-, -Se-, -N=N-, -ON=CH-, -$(R_8)N$-$N(R_8)$-, -$ON(R_8)$-, a heterocycle, or a direct bond between L and Y;

Y can be selected from -C(=O)-, -C(=S)-, -$S(O_2)$-, -S(O)-, -C(=NCN)-, -P(=O)(OR_2)-, a heteroaromatic group, or a direct bond between X and Z;

Z can be selected from -$N(R_8)$-, -O-, -S-, -Se-, -N=N-, -ON=CH-, -R_8N-NR_8-, -ONR_8-, a heterocycle, or a direct bond between Y and L;

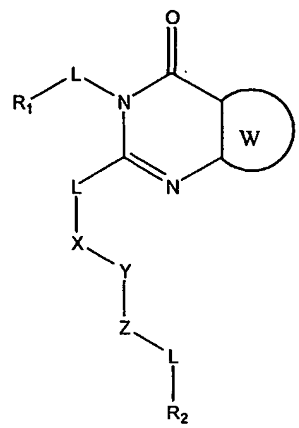
R_8, independently for each occurrence, represents H, lower alkyl, -$(CH_2)_n$aryl (e.g., substituted or unsubstituted), -$(CH_2)_n$heteroaryl (e.g., substituted or unsubstituted), or two R_8 taken together may form a 4- to 8-membered ring, e.g., with X and Z, which ring may include one or more carbonyls;

W represents a substituted or unsubsituted aryl or heteroaryl ring fused to the pyrimidone ring;

p represents, independently for each occurrence, an integer from 0 to 10, preferably from 0 to 3; and

n, individually for each occurence, represents an integer from 0 to 10, preferably from 0 to 5.

In embodiments wherein Y_1 and Z_1 are absent and X_1 comprises a pyrimidone, compounds useful in the present invention may be represented by general formula (III):



Formula III

wherein, as valence and stability permit,

R_1 and R_2, independently for each occurrence, represent H, lower alkyl, aryl (e.g., substituted or unsubstituted), aralkyl (e.g., substituted or unsubstituted, e.g., -$(CH_2)_n$aryl), or heteroaryl (e.g., substituted or unsubstituted), or heteroaralkyl (e.g., substituted or unsubstituted, e.g., -$(CH_2)_n$heteroaralkyl-);

L, independently for each occurrence, is absent or represents -$(CH_2)_n$-alkyl, -alkenyl-, -alkynyl-, -$(CH_2)_n$alkenyl-, -$(CH_2)_n$alkynyl-, -$(CH_2)_nO(CH_2)_p$-, -$(CH_2)_nNR_2(CH_2)_p$-, -$(CH_2)_nS(CH_2)_p$-, -$(CH_2)_n$alkenyl$(CH_2)_p$-, -$(CH_2)_n$alkynyl$(CH_2)_p$-, -$O(CH_2)_n$-, -$NR_2(CH_2)_n$-, or -$S(CH_2)_n$-, which may optionally be substitued with a group selected from H, substituted or unsubstituted lower alkyl,

alkenyl, or alkynyl, cycloalkylalkyl (e.g., substituted or unsubstituted, e.g., -$(CH_2)_n$cycloalkyl), (e.g., substituted or unsubstituted), aryl (e.g., substituted or unsubstituted), aralkyl (e.g., substituted or unsubstituted, e.g., -$(CH_2)_n$aryl), or heteroaryl (e.g., substituted or unsubstituted), or heteroaralkyl (e.g., substituted or unsubstituted, e.g., -$(CH_2)_n$heteroaralkyl-), preferably from H, lower alkyl, -$(CH_2)_n$aryl (e.g., substituted or unsubstituted), or -$(CH_2)_n$heteroaryl (e.g., substituted or unsubstituted);

X can be selected from -N(R_8)-, -O-, -S-, -Se-, -N=N-, -ON=CH-, -(R_8)N-N(R_8)-, -ON(R_8)-, a heterocycle, or a direct bond between L and Y;

Y can be selected from -C(=O)-, -C(=S)-, -S(O_2)-, -S(O)-, -C(=NCN)-, -P(=O)(OR_2)-, a heteroaromatic group, or a direct bond between X and Z;

Z can be selected from -N(R_8)-, -O-, -S-, -Se-, -N=N-, -ON=CH-, -R_8N-NR_8-, -ONR_8-, a heterocycle, or a direct bond between Y and L;

R_8, independently for each occurrence, represents H, lower alkyl, aryl (e.g., substituted or unsubstituted), aralkyl (e.g., substituted or unsubstituted, e.g., -$(CH_2)_n$aryl), or heteroaryl (e.g., substituted or unsubstituted), or heteroaralkyl (e.g., substituted or unsubstituted, e.g., -$(CH_2)_n$heteroaralkyl-), or two R_8 taken together may form a 4- to 8-membered ring, e.g., with X and Z, which ring may include one or more carbonyls;

W represents a substituted or unsubsituted aryl or heteroaryl ring fused to the pyrimidone ring;

p represents, independently for each occurrence, an integer from 0 to 10, preferably from 0 to 3; and

n, individually for each occurrence, represents an integer from 0 to 10, preferably from 0 to 5.

In certain embodiments, R_1 represents a substituted or unsubstituted aryl or heteroaryl group, e.g., a phenyl ring, a pyridine ring, etc. In certain embodiments

wherein -LR_1 represents a substituted aryl or heteroaryl group, R_1 is preferably not substituted with an isopropoxy (Me_2CHO-) group. In certain embodiments wherein -LR_1 represents a substituted aryl or heteroaryl group, R_1 is preferably not substituted with an ether group. In certain embodiments, substituents on R_1 (e.g., other than hydrogen) are selected from halogen, cyano, alkyl, alkenyl, alkynyl, aryl, hydroxyl, (unbranched alkyl-O-), silyloxy, amino, nitro, thiol, amino, imino, amido, phosphoryl, phosphonate, phosphine, carbonyl, carboxyl, carboxamide, anhydride, silyl, thioether, alkylsulfonyl, arylsulfonyl, sulfoxide, selenoether, ketone, aldehyde, ester, or -$(CH_2)_m$-R_8. In certain embodiments, non-hydrogen substituents are selected from halogen, cyano, alkyl, alkenyl, alkynyl, aryl, nitro, thiol, imino, amido, carbonyl, carboxyl, anhydride, thioether, alkylsulfonyl, arylsulfonyl, ketone, aldehyde, and ester. In certain embodiments, non-hydrogen substituents are selected from halogen, cyano, alkyl, alkenyl, alkynyl, nitro, amido, carboxyl, anhydride, alkylsulfonyl, ketone, aldehyde, and ester.

In certain embodiments, X can be selected from -$N(R_8)$-, -O-, -S-, a direct bond, and a heterocycle, Y can be selected from -C(=O)-, -C(=S)-, and -$S(O_2)$-, and Z can be selected from -$N(R_8)$-, -O-, -S-, a direct bond, and a heterocycle. In certain such embodiments, at least one of Z and X is present.

In certain related embodiments, X-Y-Z taken together represents a urea (NC(O)N) or an amide (NC(O) or C(O)N).

In certain embodiments, W is a substituted or unsubstituted benzene ring.

In certain embodiments, X represents a diazacarbocycle, such as a piperazine, e.g., substituted or unsubstituted.

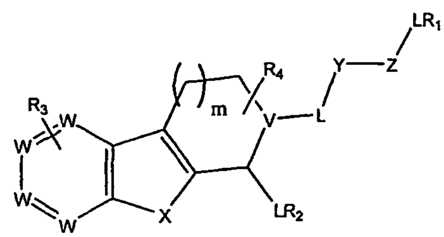
In certain embodiments, X can be selected from -$N(R_8)$-, -O-, -S-, and a direct bond, Y can be selected from -C(=O)-, -C(=S)-, and -$S(O_2)$-, and Z can be selected from -$N(R_8)$-, -O-, -S-, and a direct bond, such that at least one of X and Z is present.

In certain embodiments R_8 represents H, lower alkyl, aralkyl, heteroaralkyl, aryl, or heteroaryl, e.g., H or lower alkyl.

In certain embodiments, X represents -NH-.

In certain embodiments, -L-X- represents -(unbranched lower alkyl)-NH-, e.g., -CH_2-NH-, -CH_2CH_2-NH-, etc.

In certain other embodiments, compounds useful in the subject methods include compounds may be represented by general forumla (IV):



Formula IV

wherein, as valence and stability permit,

R_1 and R_2, independently for each occurrence, represent H, substituted or unsubstituted lower alkyl, alkenyl, or alkynyl, -$(CH_2)_n$cycloalkyl (e.g., substituted or unsubstituted), -$(CH_2)_n$aryl (e.g., substituted or unsubstituted), or -$(CH_2)_n$heterocyclyl (e.g., substituted or unsubstituted);

L, independently for each occurrence, is absent or represents -$(CH_2)_n$-alkyl, -alkenyl-, -alkynyl-, -$(CH_2)_n$alkenyl-, -$(CH_2)_n$alkynyl-, -$(CH_2)_nO(CH_2)_p$-, -$(CH_2)_nNR_2(CH_2)_p$-, -$(CH_2)_nS(CH_2)_p$-, -$(CH_2)_n$alkenyl$(CH_2)_p$-, -$(CH_2)_n$alkynyl$(CH_2)_p$-, -$O(CH_2)_n$-, -$NR_2(CH_2)_n$-, or -$S(CH_2)_n$-;

V represents N or CH;

W, independently for each occurrence, represents N or CH, such that preferably no more than one occurrence of W represents N;

X and Z, independently, can be selected from -CH-, -N(R_8)-, -O-, -S-, or -Se-;

Y can be selected from -C(=O)-, -C(=S)-, -S(O_2)-, -S(O)-, -C(=NCN)-, or -P(=O)(OR_2)-;

R_8, independently for each occurrence, represents H, substituted or unsubstituted lower alkyl, -$(CH_2)_n$cycloalkyl (e.g., substituted or unsubstituted), -$(CH_2)_n$aryl (e.g., substituted or unsubstituted), -$(CH_2)_n$heterocyclyl (e.g., substituted or unsubstituted), or

two R_8 taken together may form a 4- to 8-membered ring, e.g., with X_1 and Z_1 or X_2 and Z_1, which ring may include one or more carbonyls;

R_3 and R_4, independently represent from 1-4 substituents on the ring to which they are attached, selected from, independently for each occurrence, hydrogen, halogens, alkyls, alkenyls, alkynyls, aryls, hydroxyl, =O, =S, alkoxyl, silyloxy, amino, nitro, thiol, amines, imines, amides, phosphoryls, phosphonates, phosphines, carbonyls, carboxyls, carboxamides, anhydrides, silyls, ethers, thioethers, alkylsulfonyls, arylsulfonyls, selenoethers, ketones, aldehydes, esters, or $-(CH_2)_m-R_8$;

m represents an integer from 0-3;

p represents, independently for each occurrence, an integer from 0 to 10, preferably from 0 to 3; and

n, individually for each occurence, represents an integer from 0 to 10, preferably from 0 to 5.

In certain embodiments, R_1 and R_2 are independently selected from substituted or unsubstituted aryl, heterocyclyl, branched or unbranched alkyl, or cycloalkyl. In embodiments wherein R_1 or R_2 is aryl or heterocyclyl, substituents are preferably selected from H, alkyl, acyl, carboxy, ester, amide, cyano, ether, thioether, amino, halogen, nitro, and trihalomethyl.

In certain embodiments, R_3 is absent or represents one or two substituents selected from alkyl, acyl, carboxy, ester, amide, cyano, ether, thioether, amino, acyl, halogen, nitro, and trihalomethyl.

In certain embodiments, R_4 is absent or represents one or two substituents selected from ether, amino, thioether, alkyl, aryl, (=O), or carbonyl (e.g., carboxy, ester, ketone, aldehyde, etc.).

In certain embodiments, L is absent for each occurrence, or represents $-CH_2-$ or $-CH_2CH_2-$.

In certain embodiments, X represents NR_8. R_8 preferably represents H.

In certain embodiments, Z represents NR_8. R_8 preferably represents H.

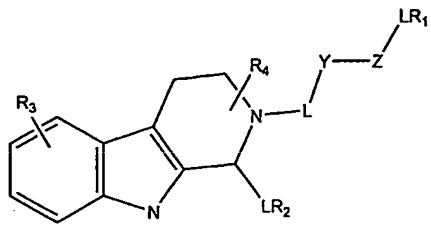
In certain embodiments, Y represents $-C(=O)-$, $-C(=S)-$, or $-S(O_2)-$.

In certain embodiments, m is 1.

In certain embodiments, W represents CH in all occurrences.

In certain embodiments, V represents N.

In certain embodiments, compounds useful in the present invention may be represented by general formula (V):



Formula V

wherein, as valence and stability permit,

R_1 and R_2, independently for each occurrence, represent H, substituted or unsubstituted lower alkyl, alkenyl, or alkynyl, -$(CH_2)_n$cycloalkyl (e.g., substituted or unsubstituted), -$(CH_2)_n$aryl (e.g., substituted or unsubstituted), or -$(CH_2)_n$heterocyclyl (e.g., substituted or unsubstituted);

L, independently for each occurrence, is absent or represents -$(CH_2)_n$-alkyl, -alkenyl-, -alkynyl-, -$(CH_2)_n$alkenyl-, -$(CH_2)_n$alkynyl-, -$(CH_2)_nO(CH_2)_p$-, -$(CH_2)_nNR_2(CH_2)_p$-, -$(CH_2)_nS(CH_2)_p$-, -$(CH_2)_n$alkenyl$(CH_2)_p$-, -$(CH_2)_n$alkynyl$(CH_2)_p$-, -$O(CH_2)_n$-, -$NR_2(CH_2)_n$-, or -$S(CH_2)_n$-;

X and Z, independently, can be selected from -CH-, -N(R_8)-, -O-, -S-, or -Se-;

Y can be selected from -C(=O)-, -C(=S)-, -S(O_2)-, -S(O)-, -C(=NCN)-, or -P(=O)(OR_2)-;

R_8, independently for each occurrence, represents H, substituted or unsubstituted lower alkyl, -$(CH_2)_n$cycloalkyl (e.g., substituted or unsubstituted), -$(CH_2)_n$aryl (e.g., substituted or unsubstituted), -$(CH_2)_n$heterocyclyl (e.g., substituted or unsubstituted), or two R_8 taken together may form a 4- to 8-membered ring, e.g., with X_1 and Z_1 or X_2 and Z_1, which ring may include one or more carbonyls;

R_3 and R_4, independently represent from 1-4 substituents on the ring to which they are attached, selected from, independently for each occurrence, hydrogen, halogens, alkyls, alkenyls, alkynyls, aryls, hydroxyl, =O, =S, alkoxyl, silyloxy, amino, nitro, thiol, amines, imines, amides, phosphoryls, phosphonates, phosphines, carbonyls, carboxyls, carboxamides, anhydrides, silyls, ethers, thioethers, alkylsulfonyls, arylsulfonyls, selenoethers, ketones, aldehydes, esters, or $-(CH_2)_m-R_8$;

p represents, independently for each occurrence, an integer from 0 to 10, preferably from 0 to 3; and

n, individually for each occurence, represents an integer from 0 to 10, preferably from 0 to 5.

In certain embodiments, R_1 and R_2 are independently selected from substituted or unsubstituted aryl, heterocyclyl, branched or unbranched alkyl, or cycloalkyl. In embodiments wherein R_1 or R_2 is aryl or heterocyclyl, substituents are preferably selected from H, alkyl, acyl, carboxy, ester, amide, cyano, ether, thioether, amino, halogen, nitro, and trihalomethyl.

In certain embodiments, R_3 is absent or represents one or two substituents selected from alkyl, acyl, carboxy, ester, amide, cyano, ether, thioether, amino, acyl, halogen, nitro, and trihalomethyl.

In certain embodiments, R_4 is absent or represents one or two substituents selected from ether, amino, thioether, alkyl, aryl, (=O), or carbonyl (e.g., carboxy, ester, ketone, aldehyde, etc.).

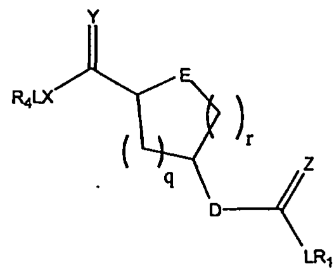
In certain embodiments, L is absent for each occurrence, or represents $-CH_2-$ or $-CH_2CH_2-$.

In certain embodiments, X represents NR_8. R_8 preferably represents H.

In certain embodiments, Z represents NR_8. R_8 preferably represents H.

In certain embodiments, Y represents -C(=O)-, -C(=S)-, or $-S(O_2)-$.

In still other embodiments, compounds which may be useful in the subject methods include compounds may be represented by general formula (VI):



Formula VI

wherein, as valence and stability permit,

R_1, R_2, R_3, and R_4, independently for each occurrence, represent H, lower alkyl, $-(CH_2)_n$aryl (e.g., substituted or unsubstituted), or $-(CH_2)_n$heteroaryl (e.g., substituted or unsubstituted);

L, independently for each occurrence, is absent or represents $-(CH_2)_n-$, -alkenyl-, -alkynyl-, $-(CH_2)_n$alkenyl-, $-(CH_2)_n$alkynyl-, $-(CH_2)_nO(CH_2)_p-$, $-(CH_2)_nNR_8(CH_2)_p-$, $-(CH_2)_nS(CH_2)_p-$, $-(CH_2)_n$alkenyl$(CH_2)_p-$, $-(CH_2)_n$alkynyl$(CH_2)_p-$, $-O(CH_2)_n-$, $-NR_8(CH_2)_n-$, or $-S(CH_2)_n-$;

X and D, independently, can be selected from $-N(R_8)-$, -O-, -S-, $-(R_8)N-N(R_8)-$, $-ON(R_8)-$, or a direct bond;

Y and Z, independently, can be selected from O or S;

E represents O, S, or NR_5, wherein R_5 represents LR_8 or $-(C{=}O)LR_8$.

R_8, independently for each occurrence, represents H, lower alkyl, $-(CH_2)_n$aryl (e.g., substituted or unsubstituted), $-(CH_2)_n$heteroaryl (e.g., substituted or unsubstituted), or two R_8 taken together may form a 4- to 8-membered ring;

p represents, independently for each occurrence, an integer from 0 to 10, preferably from 0 to 3;

n, individually for each occurrence, represents an integer from 0 to 10, preferably from 0 to 5; and

q and r represent, independently for each occurrence, an integer from 0-2.

In certain embodiments, D does not represent N-lower alkyl. In certain embodiments, D represents an aralkyl- or heteroaralkyl-substituted amine.

In certain embodiments, R_1 represents a lower alkyl group, such as a branched alkyl, a cycloalkyl, or a cycloalkylalkyl, for example, cyclopropyl, cyclopropylmethyl, neopentyl, cyclobutyl, isobutyl, isopropyl, sec-butyl, cyclobutylmethyl, etc.

In certain embodiments, Y and Z are O.

In certain embodiments, the sum of q and r is less than 4, e.g., is 2 or 3.

In certain embodiments, XLR_4, taken together, include a cyclic amine, such as a piperazine, a morpholine, a piperidine, a pyrrolidine, etc.

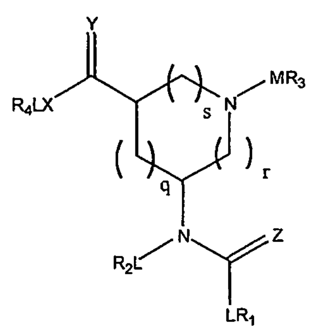
In certain embodiments, at least one of R_1, R_2, and R_3 includes an aryl or heteroaryl group. In certain related embodiments, at least two of R_1, R_2, and R_3 include an aryl or heteroaryl group. In certain embodiments, R_1 is lower alkyl.

In certain embodiments, L attached to R_1 represents O, S, or NR_8, such as NH.

In certain embodiments, E is NR_8. In certain embodiments, E represents an aralkyl- or heteroaralkyl-substituted amine, e.g., including polycyclic R_8.

In certain embodiments, X is not NH. In certain embodiments, X is included in a ring, or, taken together with –C(=Y)-, represents a tertiary amide.

In certain embodiments, compounds useful in the present invention may be represented by general formula (VII):



Formula VII

wherein, as valence and stability permit,

R_1, R_2, R_3, R_4, R_8, L, X, Y, Z, n, p, q, and r are as defined above;

M is absent or represents L, $-SO_2L-$, or -(C=O)L-; and

s represents, independently for each occurrence, an integer from 0-2.

In certain embodiments, Y and Z are O.

In certain embodiments, R_1 represents a lower alkyl group, such as a branched alkyl, a cycloalkyl, or a cycloalkylalkyl, for example, cyclopropyl, cyclopropylmethyl, neopentyl, cyclobutyl, isobutyl, isopropyl, sec-butyl, cyclobutylmethyl, etc.

In certain embodiments, the sum of q, r, and s is less than 5, e.g., is 2, 3, or 4.

In certain embodiments, XLR_4, taken together, include a cyclic amine, such as a piperazine, a morpholine, a piperidine, a pyrrolidine, etc.

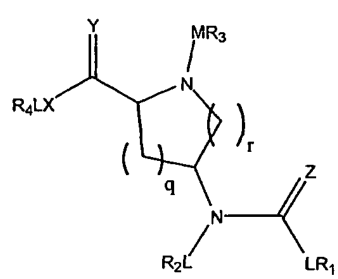
In certain embodiments, L attached to R_1 represents O, S, or NR_8, such as NH.

In certain embodiments, at least one of R_1, R_2, and R_3 includes an aryl or heteroaryl group. In certain related embodiments, at least two of R_1, R_2, and R_3 include an aryl or heteroaryl group.

In certain embodiments, M is absent.

In certain embodiments, X is not NH. In certain embodiments, X is included in a ring, or, taken together with –C(=Y)-, represents a tertiary amide.

In certain embodiments, compounds useful in the present invention may be represented by general formula (VIII):



Formula VIII

wherein, as valence and stability permit,

R_1, R_2, R_3, R_4, R_8, L, M, X, Y, Z, n, p, q, and r are as defined above.

In certain embodiments, Y and Z are O.

In certain embodiments, R_1 represents a lower alkyl group, preferably a branched alkyl, a cycloalkyl, or a cycloalkylalkyl, for example, cyclopropyl, cyclopropylmethyl, neopentyl, cyclobutyl, isobutyl, isopropyl, sec-butyl, cyclobutylmethyl, etc.

In certain embodiments, the sum of q and r is less than 4, e.g., is 2 or 3.

In certain embodiments, XLR_4, taken together, include a cyclic amine, such as a piperazine, a morpholine, a piperidine, a pyrrolidine, etc.

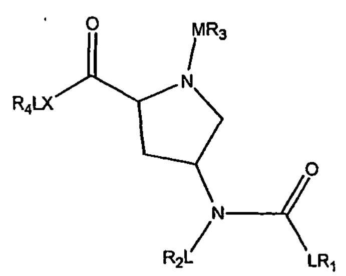
In certain embodiments, at least one of R_1, R_2, and R_3 includes an aryl or heteroaryl group. In certain related embodiments, at least two of R_1, R_2, and R_3 include an aryl or heteroaryl group. In certain embodiments, R_1 is lower alkyl.

In certain embodiments, L attached to R_1 represents O, S, or NR_8, such as NH.

In certain embodiments, M is absent.

In certain embodiments, X is not NH. In certain embodiments, X is included in a ring, or, taken together with –C(=Y)-, represents a tertiary amide.

In certain embodiments, compounds useful in the present invention may be represented by general formula (IX):



Formula IX

wherein, as valence and stability permit,

R_1, R_2, R_3, R_4, R_8, L, M, X, n, and p are as defined above.

In certain embodiments, XLR_4, taken together, include a cyclic amine, such as a piperazine, a morpholine, a piperidine, a pyrrolidine, etc.

In certain embodiments, R_1 represents a lower alkyl group, preferably a branched alkyl, a cycloalkyl, or a cycloalkylalkyl, for example, cyclopropyl, cyclopropylmethyl, neopentyl, cyclobutyl, isobutyl, isopropyl, sec-butyl, cyclobutylmethyl, etc.

In certain embodiments, at least one of R_1, R_2, and R_3 includes an aryl or heteroaryl group. In certain related embodiments, at least two of R_1, R_2, and R_3 include an aryl or heteroaryl group. In certain embodiments, R_1 is lower alkyl.

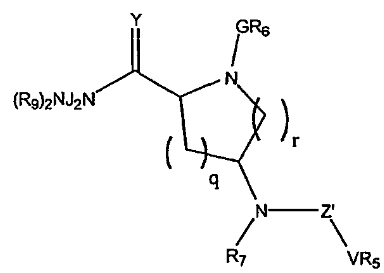
In certain embodiments, L attached to R_1 represents O, S, or NR_8, such as NH.

In certain embodiments, M is absent.

In certain embodiments, X is not NH. In certain embodiments, X is included in a ring, or, taken together with –C(=Y)-, represents a tertiary amide.

In certain embodiments L represents a direct bond for all occurrences.

In certain embodiments, compounds useful in the present invention may be represented by general formula (X):



Formula X

wherein, as valence and stability permit,

Y, n, p, q, and r are as defined above;

Z' represents -C(=O)-, -C(=S)-, -C(=NH)-, SO_2, or SO, preferably -C(=O)-, -C(=S)-;

V is absent or represents O, S, or NR_8;

G is absent or represents –C(=O)- or $-SO_2-$;

J, independently for each occurrence, represents H or substituted or unsubstituted lower alkyl or alkylene, such as methyl, ethyl, methylene, ethylene, etc., attached to NC(=Y), such that both occurrences of N adjacent to J are linked through at least one occurrence of J, and

R_9, independently for each occurrence, is absent or represents H or lower alkyl, or two occurrences of J or one occurrence of J taken together with one occurrence of R_9, forms a ring of from 5 to 7 members, which ring includes one or both occurrences of N;

R_5 represents substituted or unsubstituted alkyl (e.g., branched or unbranched), alkenyl (e.g., branched or unbranched), alkynyl (e.g., branched or unbranched), cycloalkyl, or cycloalkylalkyl;

R_6 represents substituted or unsubstituted aryl, aralkyl, heteroaryl, heteroaralkyl, heterocyclyl, heterocyclylalkyl, cycloalkyl, or cycloalkylalkyl, including polycyclic groups; and

R_7 represents substituted or unsubstituted aryl, aralkyl, heteroaryl, or heteroaralkyl.

In certain embodiments, Y is O. In certain embodiments, Z' represents SO_2, -C(=O)-, or -C(=S)-.

In certain embodiments, the sum of q and r is less than 4.

In certain embodiments, NJ_2N, taken together, represent a cyclic diamine, such as a piperazine, etc., which may be substituted or unsubstituted, e.g., with one or more substitutents such as oxo, lower alkyl, lower alkyl ether, etc. In certain other embodiments, NJ_2 or NJR_9 taken together represent a substituted or unsubstituted heterocyclic ring to which the other occurrence of N is attached. In certain embodiments, one or both occurrences of J are substituted with one or more of lower alkyl, lower alkyl ether, lower alkyl thioether, amido, oxo, etc. In certain embodiments, a heterocyclic ring which comprises an occurrence of J has from 5 to 8 members.

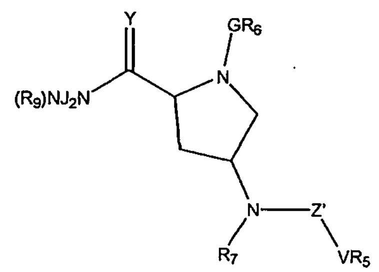
In certain embodiments, R_5 represents a branched alkyl, cycloalkyl, or cycloalkylalkyl.

In certain embodiments, R_6 includes at least one heterocyclic ring, such as a thiophene, furan, oxazole, benzodioxane, benzodioxole, pyrrole, indole, etc.

In certain embodiments, R_7 represents a phenyl alkyl, such as a benzyl group, optionally substituted with halogen, hydroxyl, lower alkyl, nitro, cyano, lower alkyl ether (e.g., optionally substituted, such as CHF_2CF_2O), or lower alkyl thioether (e.g., optionally substituted, such as CF_3S).

In certain embodiments, R_8, when it occurs in V, represents H or lower alkyl, preferably H.

In certain embodiments, compounds useful in the present invention may be represented by general formula (XI):



Formula XI

wherein, as valence and stability permit,

R_5, R_6, R_7, R_8, R_9, R_{10}, G, J, V, Y, Z', n, and p are as defined above.

In certain embodiments, Y is O. In certain embodiments, Z' represents SO_2, -C(=O)-, or -C(=S)-.

In certain embodiments, NJ_2N, taken together, represent a heterocyclic ring, such as a piperazine, etc., which may be substituted or unsubstituted, e.g., with one or more substitutents such as oxo, lower alkyl, lower alkyl ether, etc. In certain other embodiments, NJ_2 or NJR_9 taken together represent a substituted or unsubstituted heterocyclic ring to which the other occurrence of N is attached. In certain embodiments, one or both occurrences of J are substituted with one or more of lower

alkyl, lower alkyl ether, lower alkyl thioether, amido, oxo, etc. In certain embodiments, a heterocyclic ring which comprises an occurrence of J has from 5 to 8 members.

In certain embodiments, R_5 represents a branched alkyl, cycloalkyl, or cycloalkylalkyl.

In certain embodiments, R_6 includes at least one heterocyclic ring, such as a thiophene, furan, oxazole, benzodioxane, benzodioxole, pyrrole, indole, etc.

In certain embodiments, R_7 represents a phenyl alkyl, such as a benzyl group, optionally substituted with halogen, hydroxyl, lower alkyl, nitro, cyano, lower alkyl ether (e.g., optionally substituted, such as CHF_2CF_2O), or lower alkyl thioether (e.g., optionally substituted, such as CF_3S).

In certain embodiments, R_8, when it occurs in V, represents H or lower alkyl, preferably H.

In certain preferred embodiments, the subject inhibitors inhibit *hedgehog*-mediated signal transduction with an IC_{50} of 1 mM or less, more preferably of 1 μM or less, and even more preferably of 1 nM or less.

Moreover, the subject methods can be performed on cells which are provided in culture (*in vitro*), or on cells in a whole animal (*in vivo*). See, for example, PCT publications WO 95/18856 and WO 96/17924 (the specifications of which are expressly incorporated by reference herein).

V. Agonists of Hedgehog Biological Activity

Preferred hedgehog therapeutics useful in methods of the invention are agonists that are derived from several sources of hedgehog protein. In one embodiment, the agonist is not N-terminally clipped (as described above). Other embodiments of a hedgehog therapeutic suitable for the present methods are based, in part, on the discovery disclosed in U.S. Patent Application No. 60/067,423 (12/3/97:PCT Publication that human Sonic hedgehog, expressed as a full-length construct in either insect or in mammalian cells, has a hydrophobic palmitoyl group appended to the alpha-amine of the N-terminal cysteine. This is the first example of an extracellular signaling protein being modified in such a manner, and, in contrast to thiol-linked palmitic acid

modifications whose attachment is readily reversible, this novel N-linked palmitoyl moiety is likely to be very stable by analogy with myristic acid modifications.

The agonists have at least one of the following properties: (i) the isolated protein binds the receptor patched-1 with an affinity that is at similar to, but is preferably higher than, the binding of mature hedgehog protein to patched-1; or (ii) the isolated protein binds to a hedgehog protein in such a way as to increase the proteins binding affinity to patched-1 when tested in an in vitro CH310T1/2 cell-based AP induction assay. Agonists of the invention may also have the additional properties of being (iii) able to solely induce ptc-1 and gli-1 expression.

The preferred agonists for use in conjugation with a non-hedgehog conjugate (e.g., immunoglobulin or fragment thereof) include a derivitized hedgehog polypeptide sequence as well as other N-terminal and/or C-terminal amino acid sequence or it may include all or a fragment of a hedgehog amino acid sequence. Agonist polypeptides of the invention include those that arise as a result of the existence of multiple genes, alternative transcription events, alternative RNA splicing events, and alternative translational and posttranslational events. The polypeptide can be made entirely by synthetic means or can be expressed in systems, e.g., cultured cells, which result in substantially the same posttranslational modifications present when the protein is expressed in a native cell, or in systems which result in the omission of posttranslational modifications present when expressed in a native cell.

In one embodiment, the agonist is a hedgehog polypeptide with one or more of the following characteristics:

(i) it has at least 30, 40, 42, 50, 60, 70, 80, 90 or 95% sequence identity with a hedgehog sequence such as SEQ ID NOS: 10-18 or 23-26;

(ii) it has a cysteine or a functional equivalent as the N-terminal end;

(iii) it may induce alkaline phosphatase activity in C3H10T1/2 cells;

(iv) it has an overall sequence identity of at least 50%, preferably at least 60%, more preferably at least 70, 80, 90, or 95%, with a polypeptide of a hedgehog sequence;

(v) it can be isolated from natural sources such as mammalian cells;

(vi) it can bind or interact with patched; and

(vii) it may be hydrophobically-modified (i.e., it has at least one hydrophobicmoiety attached to the polypeptide).

Increasing the overall hydrophobic nature of a hedgehog protein increases the biological activity of the protein. The potency of a signaling protein such as hedgehog can be increased by: (a) chemically modifying, such as by adding a hydrophobic moiety to, the sulfhydryl and/or to the alpha-amine of the N-terminal cysteine (see U.S.60/067,423); (b) replacing the N-terminal cysteine with a hydrophobic amino acid (see U.S. 60/067,423); or (c) replacing the N-terminal cysteine with a different amino acid and then chemically modifying the substituted residue so as to add a hydrophobic moiety at the site of the substitution.

Additionally, modification of a hedgehog protein at an internal residue on the surface of the protein with a hydrophobic moiety by: (a) replacing the internal residue with a hydrophobic amino acid; or (b) replacing the internal residue with a different amino acid and then chemically modifying the substituted residue so as to add a hydrophobic moiety at the site of the substitution will retain or enhance the biological activity of the protein.

Additionally, modification of a protein such as a hedgehog protein at the C-terminus with a hydrophobic moiety by: (a) replacing the C-terminal residue with a hydrophobic amino acid; or (b) replacing the C-terminal residue with a different amino acid and then chemically modifying the substituted residue so as to add a hydrophobic moiety at the site of the substitution, will retain or enhance the biological activity of the protein.

For hydrophobically-modified hedgehog obtained by chemically modifying the soluble, unmodified protein, palmitic acid and other lipids can be added to soluble Shh to create a lipid-modified forms with increased potency in the C3HIOT1/2 assay. Another form of protein encompassed by the invention is a protein derivatized with a variety of lipid moieties. The principal classes of lipids that are encompassed within this invention are fatty acids and sterols (e.g., cholesterol). Derivatized proteins of the

invention contain fatty acids which are cyclic, acyclic (i.e., straight chain), saturated or unsaturated, mono-carboxylic acids. Exemplary saturated fatty acids have the generic formula: CH3 (CH2)n COON. Table 2 below lists examples of some fatty acids that can be derivatized conveniently using conventional chemical methods.

TABLE 2: Exemplary Saturated and Unsaturated Fatty Acids

Saturated Acids: CH3 (CH2)n COOH:

Value of n	Common Name
2	butyric acid
4	caproic acid
6	caprylic acid
8	capric acid
10	lauric acid
12	myristic acid*
14	palmitic acid*
16	stearic acid*
18	arachidic acid*
20	behenic acid
22	lignoceric acid

Unsaturated Acids:

CH3CH=CHCOOH	crotonic acid
CH3(CH2)3CH=CH(CH2)7COOH	myristoleic acid*
CH3(CH2)5CH=CH (CH2)7COOH	palmitoleic acid*
CH3(CH2)7CH=CH(CH2)7COOH	oleic acid*
CH3(CH2)3(CH2CH=CH)2(CH2)7COOH	linoleic acid
CH3(CH2CH=CH)3(CH2)7COOH	linolenic acid
CH3(CH2)3(CH2CH=CH)4(CH2)3COOH	arachidonic acid

The asterisk (*) denotes fatty acids detected in recombinant hedgehog protein secreted from a soluble construct (Pepinsky et al., supra).

Other lipids that can be attached to the protein include branched-chain fatty acids and those of the phospholipid group such as the phosphatidylinositols (i.e., phosphatidylinositol 4-monophosphate and phosphatidylinositol 4,5- biphosphate), phosphatidycholine, phosphatidylethanolamine, phosphatidylserine, and isoprenoids such as farnesyl or geranyl groups. Lipid-modified hedgehog proteins can be purified from either a natural source, or can be obtained by chemically modifying the soluble, unmodified protein.

For protein purified from a natural source, we showed that when full-length human Sonic hedgehog (Shh) was expressed in insect cells and membrane-bound Shh purified from the detergent-treated cells using a combination of SP-Sepharose chromatography and immunoaffinity chromatography, that the purified protein migrated on reducing SDS-PAGE gels as a single sharp band with an apparent mass of 20 kDa. See PCT The soluble and membrane-bound Shh proteins were readily distinguishable by reverse phase HPLC, where the tethered forms eluted later in the acetonitrile gradient. We then demonstrated that human Sonic hedgehog is tethered to cell membranes in two forms, one form that contains a cholesterol, and therefore is analogous to the data reported previously for Drosophila hedgehog, and a second novel form that contains both a cholesterol and a palmitic acid modification. Both modified forms were equally as active in the C3H10T1/2 alkaline phosphatase assay, but both were about 30-times more potent than soluble human Shh lacking the tether(s). The hydrophobic modifications did not significantly affect the apparent binding affinity of Shh for its receptor, patched.

For specific lipid-modified hedgehog obtained by chemically modifying the soluble, unmodified protein, palmitic acid and other lipids can be added to soluble Shh to create a lipid-modified forms with increased potency in the C3H10T1/2 assay. Generally, therefore, the reactive lipid moiety can be in the form of thioesters of saturated or unsaturated carboxylic acids such as a Coenzyme A thioesters. Such materials and their derivatives may include, for example, commercially available Coenzyme A derivatives such as palmitoleoyl Coenzyme A, arachidoyl Coenzyme A,

arachidonoyl Coenzyme A, lauroyl Coenzyme A and the like. These materials are readily available from Sigma Chemical Company (St. Louis, MO., 1998 catalog pp. 303-306).

There are a wide range of hydrophobic moieties with which hedgehog polypeptides can be derivatived. A hydrophobic group can be, for example, a relatively long chain alkyl or cycloalkyl (preferably n-alkyl) group having approximately 7 to 30 carbons. The alkyl group may terminate with a hydroxy or primary amine "tail". To further illustrate, such molecules include naturally-occurring and synthetic aromatic and non-aromatic moieties such as fatty acids, esters and alcohols, other lipid molecules, cage structures such as adamantane and buckminsterfullerenes, and aromatic hydrocarbons such as benzene, perylene, phenanthrene, anthracene, naphthalene, pyrene, chrysene, and naphthacene.

Particularly useful as hydrophobic molecules are alicyclic hydrocarbons, saturated and unsaturated fatty acids and other lipid and phospholipid moieties, waxes, cholesterol, isoprenoids, terpenes and polyalicyclic hydrocarbons including adamantane and buckminsterfullerenes, vitamins, polyethylene glycol or oligoethylene glycol, (C1-C18)-alkyl phosphate diesters, -O-CH2-CH(OH)-O-(C 12-C 18)-alkyl, and in particular conjugates with pyrene derivatives. The hydrophobic moiety can be a lipophilic dye suitable for use in the invention include, but are not limited to, diphenylhexatriene, Nile Red, N-phenyl-l-naphthylamine, Prodan, Laurodan, Pyrene, Perylene, rhodamine, rhodamine B, tetramethylrhodamine, Texas Red, sulforhodamine, 1,1'-didodecyl-3,3,3',3'tetramethylindocarbocyanine perchlorate, octadecyl rhodamine B and the BODIPY dyes available from Molecular Probes Inc.

Other exemplary lipophilic moieties include aliphatic carbonyl radical groups include 1- or 2-adamantylacetyl, 3-methyladamant-1-ylacetyl, 3-methyl-3-bromo-l-adamantylacetyl, 1-decalinacetyl, camphoracetyl, camphaneacetyl, noradamantylacetyl, norbornaneacetyl, bicyclo[2.2.2.]-oct-5-eneacetyl, 1-methoxybicyclo[2.2.2.]-oct-5-ene-2-carbonyl, cis-5-norbornene-endo-2,3-dicarbonyl, 5-norbornen-2-ylacetyl, (1R)-(-)-myrtentaneacetyl, 2-norbornaneacetyl, anti-3-oxo-tricyclo[2.2.1.0<2,6>]-heptane-7-carbonyl, decanoyl, dodecanoyl, dodecenoyl, tetradecadienoyl, decynoyl or dodecynoyl.

1. Chemical Modifications of the N-terminal cysteine of hedgehog

If an appropriate amino acid is not available at a specific position, site-directed mutagenesis can be used to place a reactive amino acid at that site. Reactive amino acids include cysteine, lysine, histidine, aspartic acid, glutamic acid, serine, threonine, tyrosine, arginine, methionine, and tryptophan. Mutagenesis could also be used to place the reactive amino acid at the N- or C-terminus or at an internal position.

For example, it is possible to chemically modify an N-terminal cysteine of a biologically active protein, such as a hedgehog protein, or eliminate the N-terminal cysteine altogether and still retain the protein's biological activity. The replacement or modification of the N-terminal cysteine of hedgehog with a hydrophobic amino acid results in a protein with increased potency in a cell-based signaling assay. By replacing the cysteine, this approach eliminates the problem of suppressing other unwanted modifications of the cysteine that can occur during the production, purification, formulation, and storage of the protein. The generality of this approach is supported by the finding that three different hydrophobic amino acids, phenylalanine, isoleucine, and methionine, each give a more active form of hedgehog, and thus, an agonist.

This is also important for conjugation with non-hedgehog moieties (e.g., immunoglobulin) as described below in which we introduce two isoleucine residues to the N-terminal cysteine end of Sonic and Desert hedgehog. This effectively allows us to use the thiol of C-terminal cysteine as the reactive site for covalent coupling. Thus, replacement of the N-terminal cysteine with any other hydrophobic amino acid should result in an active protein. Furthermore, since we have found a correlation between the hydrophobicity of an amino acid or chemical modification and the potency of the corresponding modified protein in the C3HIOT1/2 assay (e.g. Phe > Met, long chain length fatty acids > short chain length), it could be envisioned that adding more than one hydrophobic amino acid to the hedgehog sequence would increase the potency of the agonist beyond that achieved with a single amino acid addition. Indeed, addition of two consecutive isoleucine residues to the N-terminus of human Sonic hedgehog results in an increase in potency in the C3HIOT1/2 assay as compared to the mutant with only a

single isoleucine added. Thus, adding hydrophobic amino acids at the N- or C-terminus of a hedgehog protein, in a surface loop, or some combination of positions would be expected to give a more active form of the protein. The substituted amino acid need not be one of the 20 common amino acids. Methods have been reported for substituting unnatural amino acids at specific sites in proteins and this would be advantageous if the amino acid was more hydrophobic in character, resistant to proteolytic attack, or could be used to further direct the hedgehog protein to a particular site in vivo that would make its activity more potent or specific. Unnatural amino acids can be incorporated at specific sites in proteins during in vitro translation, and progress is being reported in creating in vivo systems that will allow larger scale production of such modified proteins.

There are many modifications of the N-terminal cysteine which protect the thiol and append a hydrophobic moiety. One of skill in the art is capable of determining which modification is most appropriate for a particular therapeutic use. Factors affecting such a determination include cost and ease of production, purification and formulation, solubility, stability, potency, pharmacodynamics and kinetics, safety, immunogenicity, and tissue targeting.

2. Chemical modification of other amino acids.

There are specific chemical methods for the modification of many other amino acids. Therefore, another route for synthesizing a more active form of hedgehog would be to chemically attach a hydrophobic moiety to an amino acid in hedgehog other than to the N-terminal cysteine. If an appropriate amino acid is not available at the desired position, site-directed mutagenesis could be used to place the reactive amino acid at that site in the hedgehog structure, whether at the N- or C-terminus or at another position. Reactive amino acids would include cysteine, lysine, histidine, aspartic acid, glutamic acid, serine, threonine, tyrosine, arginine, methionine, and tryptophan. Thus the goal of creating a better hedgehog agonist could be attained by many chemical means and we do not wish to be restricted by a particular chemistry or site of modification since our results support the generality of this approach.

The hedgehog polypeptide can be linked to the hydrophobic moiety in a number of ways including by chemical coupling means, or by genetic engineering. To illustrate, there are a large number of chemical cross-linking agents that are known to those skilled in the art. For the present invention, the preferred cross-linking agents are heterobifunctional cross-linkers, which can be used to link the hedgehog polypeptide and hydrophobic moiety in a stepwise manner. Heterobifunctional cross-linkers provide the ability to design more specific coupling methods for conjugating to proteins, thereby reducing the occurrences of unwanted side reactions such as homo-protein polymers. A wide variety of heterobifunctional cross-linkers are known in the art. These include: succinimidyl 4-(N-maleimidomethyl) cyclohexane- 1-carboxylate (SMCC), m-Maleimidobenzoyl-N- hydroxysuccinimide ester (MBS); N-succinimidyl (4-iodoacetyl) aminobenzoate (SIAB), succinimidyl 4-(p-maleimidophenyl) butyrate (SMPB), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC); 4-succinimidyloxycarbonyl- a-methyl-a-(2-pyridyldithio)-tolune (SMPT), N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), succinimidyl 6-[3-(2-pyridyldithio) propionate] hexanoate (LC-SPDP). Those cross-linking agents having N-hydroxysuccinimide moieties can be obtained as the N-hydroxysulfosuccinimide analogs, which generally have greater water solubility. In addition, those cross-linking agents having disulfide bridges within the linking chain can be synthesized instead as the alkyl derivatives so as to reduce the amount of linker cleavage in vivo.

One particularly useful class of heterobifunctional cross-linkers, included above, contain the primary amine reactive group, N-hydroxysuccinimide (NHS), or its water soluble analog N-hydroxysulfosuccinimide (sulfo-NHS). Primary amines (lysine epsilon groups) at alkaline pH's are unprotonated and react by nucleophilic attack on NHS or sulfo-NHS esters. This reaction results in the formation of an amide bond, and release of NHS or sulfo-NHS as a by-product.

Another reactive group useful as part of a heterobifunctional cross-linker is a thiol reactive group. Common thiol reactive groups include maleimides, halogens, and pyridyl disulfides. Maleimides react specifically with free sulfhydryls (cysteine residues) in minutes, under slightly acidic to neutral (pH 6.5-7.5) conditions. Halogens

(iodoacetyl functions) react with -SH groups at physiological pH's. Both of these reactive groups result in the formation of stable thioether bonds.

Generally, the structure of an agonistic hedgehog therapeutic useful in this invention is a chimeric molecule that has the general formula: X-Y-Z, where wherein X is a polypeptide having the amino acid sequence, or portion thereof, consisting of the amino acid sequence of hedgehog; Y is an optional linker moiety; and Z is a polypeptide comprising at least a portion of a polypeptide other than hedgehog . Preferably, X includes at least a biologically active N-terminal fragment of is human Sonic, Indian or Desert hedgehog. In the more preferred embodiments, Z is a protein with an 19-like constant and/or variable domain. Most preferably, Z is at least a portion of a constant region of an immunoglobulin and can be derived from an immunoglobulin of the class selected from IgM, IgG, IgD, IgA, and IgE. If the class is IgG, then it is selected from one of IgG 1, IgG2, IgG3 and IgG4. The constant region of human IgM and IgE contain 4 constant regions (CHI, (hinge), CH2, CH3 and CH4, whereas the constant region of human IgG, IgA and IgD contain 3 constant regions (CHI, (hinge), CH2 and CH3. In the most preferred fusion proteins of the invention, the constant region contains at least the hinge, CH2 and CH3 domains.

In another embodiment, the chimeric molecule has the structure D-[Sp]-B-[Sp]-C, where D is a non-hedgehog moiety such as described herein; [Sp] is an optional spacer peptide sequence; B is a hedgehog protein (which optionally may be a mutein as described herein); and C is an optional hydrophobic moiety linked (optionally by way of the spacer peptide) to the hedgehog protein D or another residue such as a surface site of the protein.

The present invention provides for multimeric hedgehog therapeutic molecules. Such multimers may be generated by using those Fc regions, or portions thereof, of Ig molecules which are usually multivalent such as IgM pentamers or IgA dimers. It is understood that a J chain polypeptide may be needed to form and stabilize IgM pentamers and IgA dimers. Alternatively, multimers of hedgehog therapeutic proteins may be formed using a protein with an affinity for the Fc region of Ig molecules, such as

Protein A. For instance, a plurality of hedgehog / immunoglobulin fusion proteins may be bound to Protein A-agarose beads.

These multivalent forms are useful since they possess multiple hedgehog receptor binding sites. For example, a bivalent soluble hedgehog therapeutic may consist of two tandem repeats of those amino acids encoded by nucleic acids of SEQ. ID NOS: 1-9 or 21, 22 or 27 (moiety X in the generic formula) separated by a linker region (moiety Y), the repeats bound to at least a portion of an immunoglobulin constant domain (moiety Z). Alternate polyvalent forms may also be constructed, for example, by chemically coupling chimeric hedgehog therapeutics of the invention to any clinically acceptable carrier molecule, a polymer selected from the group consisting of Ficoll, polyethylene glycol or dextran using conventional coupling techniques. Alternatively, hedgehog may be chemically coupled to biotin, and the biotin-hedgehog chimera then allowed to bind to avidin, resulting in tetravalent avidin/biotin/hedgehog molecules. Chimeric hedgehog proteins may also be covalently coupled to dinitrophenol (DNP) or trinitrophenol (TNP) and the resulting conjugate precipitated with anti-DNP or anti-TNP-IgM, to form decameric conjugates with a valency of 10 for hedgehog receptor binding sites

Polymer Conjugates of Hedgehog Therapeutics

One unique property of polyalkylene glycol-derived polymers of value for therapeutic applications of the present invention is their general biocompatibility. These polymers have various water solubility properties and are not toxic. They are believed non-immunogenic and non-antigenic and do not interfere with the biological activities of the hedgehog protein moiety when conjugated under the conditions described herein. They have long circulation in the blood and are easily excreted from living organisms.

Hedgehog therapeutics are conjugated most preferably via a terminal reactive group on the polyalkylene glycol polymer although conjugations can also be branched from non-terminal reactive groups. The polymer with the reactive group(s) is designated herein as "activated polymer". The reactive group would be expected to selectively react with free amino or other reactive groups on the hedgehog protein. In theory, the

activated polymer(s) are reacted so that attachment could occur at any available hedgehog amino group such as alpha amino groups or the epsilon-amino groups of lysines, or -SH groups of cysteines. Free carboxylic groups, suitably activated carbonyl groups, hydroxyl, guanidyl, oxidized carbohydrate moieties and mercapto groups of the hedgehog protein (if available) can also be used as attachment sites.

In particular, the chemical modification of any N-terminal cysteine to protect the thiol, with concomitant conjugation with a polyalkylene glycol moiety (i.e., PEG), can be carried out in numerous ways by someone skilled in the art. See United States Patent 4,179,337. The sulfhydryl moiety, with the thiolate ion as the active species, is the most reactive functional group in a protein. There are many reagents that react faster with the thiol than any other groups. See Chemistry of Protein Conjugation and Cross-Linking (S. S. Wong, CRC Press, Boca Raton, FL, 1991). The thiol of an N-terminal cysteine, such as found in all hedgehog proteins, would be expected to be more reactive than internal cysteines within the sequence. This is because the close proximity to the alpha-amine will lower the pKa of the thiol resulting in a greater degree of proton dissociation to the reactive thiolate ion at neutral or acid pH. In addition, the cysteine at the N-terminus of the structure is more likely to be exposed than the other two cysteines in the hedgehog sequence that are found buried in the protein structure.

Other examples of methods that provide linkage between a polyalkylene glycol and the N-terminal cysteine would be reactions with other alpha-haloacetyl compounds, organomercurials, disulfide reagents, and other N-substituted maleimides. Numerous derivatives of these active species are available commercially (e.g., ethyl iodoacetate (Aldrich, Milwaukee WI), phenyl disulfide (Aldrich), and N-pyrenemaleimide (Molecular Probes, Eugene OR)) or could be synthesized readily (e.g., N-alkyliodoacetamides, N-alkylmaleimides, and organomercurials). Another aspect to the reactivity of an N-terminal cysteine is that it can take part in reaction chemistries unique to its 1,2-aminothiol configuration. One example is the reaction with thioester groups to form an N-terminal amide group via a rapid S to N shift of the thioester. This reaction chemistry can couple together synthetic peptides and can be used to add single or multiple, natural or unnatural, amino acids or other hydrophobic groups via the

appropriately activated peptide. Another example, is the reaction with aldehydes to form the thiazolidine adduct. Numerous hydrophobic derivatives of thiol esters (e.g., C2-C24 saturated and unsaturated fatty acyl Coenzyme A esters (Sigma Chemical Co., St. Louis MO)), aldehydes (e.g., butyraldehyde, n-decyl aldehyde, and n-myristyl aldehyde (Aldrich)), and ketones (e.g., 2-, 3-, and 4-decanone (Aldrich)) are available commercially or could be synthesized readily. In a similar manner, thiomorpholine could be prepared from a variety of alpha-haloketone starting materials.

Several observations suggest that the C-terminus or amino acids near the C-terminus would be preferred targets for modification with a polyalkylene glycol moiety. Briefly, we have shown that: (i) The wild-type protein is naturally modified with cholesterol at the C-terminus, indicating that it is exposed and available for modification. Indeed, we showed that treatment with thrombin results in selective release of the C-terminal 3 amino acids (See U.S.S.N. 60/106,703, filed 11/2/98, now PCT Number -incorporated herein by reference); (ii) We performed extensive SAR analyses and discovered that the C-terminal 11 amino acids could be deleted without harmful effects on folding or function; (iii) We have made hedgehog/Ig fusion proteins by attaching an Ig moiety to the C-terminus of hedgehog without harmful effects on folding or function (data not presented here).

While there is no simple chemical strategy for targeting a polyalkylene glycol polymer such as PEG to the C-terminus of hedgehog, it is straightforward to genetically engineer a site that can be used to target the polymer moiety, as discussed above with regard to site-directed mutagenesis. For example, incorporation of a Cys at a site that is at or near the C-terminus allows specific modification using a maleimide, vinylsulfone or haloacetate- activated polyalkylene glycol (e.g., PEG). As discussed above in Section A, these derivatives can be used specifically for modification of the engineered C-terminal cysteines due to the high selectively of these reagents for Cys. Other strategies such as incorporation of a histidine tag which can be targeted (Fancy et al., (1996) Chem. & Biol. 3: 551) or an additional glycosylation site, represent other alternatives for modifying the C-terminus of hedgehog. A single polymer molecule may be employed for conjugation with the hedgehog protein and modified versions thereof as

discussed above, although it is also contemplated that more than one polymer molecule can be attached as well. Conjugated hedgehog compositions of the invention may find utility in both in vivo as well as non-in vivo applications. Additionally, it will be recognized that the conjugating polymer may utilize any other groups, moieties, or other conjugated species, as appropriate to the end use application. By way of example, it may be useful in some applications to covalently bond to the polymer a functional moiety imparting UV-degradation resistance, or antioxidation, or other properties or characteristics to the polymer. As a further example, it may be advantageous in some applications to functionalize the polymer to render it reactive or cross-linkable in character, to enhance various properties or characterisics of the overall conjugated material. Accordingly, the polymer may contain any functionality, repeating groups, linkages, or other constitutent structures which do not preclude the efficacy of the conjugated hedgehog composition for its intended purpose. Other objectives and advantages of the present invention will be more fully apparent from the ensuing disclosure and appended claims.

Illustrative polymers that may usefully be employed to achieve these desirable characteristics are described herein below in exemplary reaction schemes. In covalently bonded peptide applications, the polymer may be functionalized and then coupled to free amino acid(s) of the peptide(s) to form labile bonds.

Generally from about 1.0 to about 10 moles of activated polymer per mole of protein is employed, depending on the particular reaction chemistry and the protein concentration. The final amount is a balance between maximizing the extent of the reaction while minimizing non-specific modifications of the product and, at the same time, defining chemistries that will maintain optimum activity, while at the same time optimizing, if possible, the half-life of the protein. Preferably, at least about 50% of the biological activity of the protein is retained, and most preferably 100% is retained.

The reactions may take place by any suitable method used for reacting biologically active materials with inert polymers. Generally the process involves preparing an activated polymer (that may have at least one terminal hydroxyl group) and thereafter reacting the protein with the activated polymer to produce the soluble protein

suitable for formulation. The above modification reaction can be performed by several methods, which may involve one or more steps.

Suitable methods of attaching a polyalkylene glycol moiety to a C-terminal cysteine involve using such moieties that are activated with a thiol reactive group, as generally discussed above. Common thiol reactive groups include maleimides, vinylsulfones or haloacetates. These derivatives can be used specifically for modification of cysteines due to the high selectively of these reagents for -SH. Maleimides react specifically with free sulfhydryls (cysteine residues) in minutes, under slightly acidic to neutral (pH 6.0-7.5) conditions. This pH range is preferred although the reaction will proceed, albeit slowly, at pH 5.0. Halogens (iodoacetyl functions) react with -SH groups at physiological pH's to slightly basic conditions. Both of these reactive groups result in the formation of stable thioether bonds.

In the practice of the methods of the present invention, polyalkylene glycol residues of C1-C4 alkyl polyalkylene glycols, preferably polyethylene glycol (PEG), or poly(oxy)alkylene glycol residues of such glycols are advantageously incorporated in the polymer systems of interest. Thus, the polymer to which the protein is attached can be a homopolymer of polyethylene glycol (PEG) or is a polyoxyethylated polyol, provided in all cases that the polymer is soluble in water at room temperature. Non-limiting examples of such polymers include polyalkylene oxide homopolymers such as PEG or polypropylene glycols, polyoxyethylenated glycols, copolymers thereof and block copolymers thereof, provided that the water solubility of the block copolymer is maintained. Examples of polyoxyethylated polyols include, for example, polyoxyethylated glycerol, polyoxyethylated sorbitol, polyoxyethylated glucose, or the like. The glycerol backbone of polyoxyethylated glycerol is the same backbone occurring naturally in, for example, animals and humans in mono-, di-, and triglycerides. Therefore, this branching would not necessarily be seen as a foreign agent in the body.

As an alternative to polyalkylene oxides, dextran, polyvinyl pyrrolidones, polyacrylamides, polyvinyl alcohols, carbohydrate-based polymers and the like may be used. Moreover, heteropolymers (i.e., polymers consisting of more than one species of

monomer such as a copolymer) as described in U.S. Patent 5,359,030 may be used (e.g., proteins conjugated to polymers comprising a polyalkylene glycol moiety and one or more fatty acids) Those of ordinary skill in the art will recognize that the foregoing list is merely illustrative and that all polymer materials having the qualities described herein are contemplated. The polymer need not have any particular molecular weight, but it is preferred that the molecular weight be between about 300 and 100,000, more preferably between 10,000 and 40,000. In particular, sizes of 20,000 or more are best at preventing protein loss due to filtration in the kidneys. Moreover, in another aspect of the invention, one can utilize hedgehog covalently bonded to the polymer component in which the nature of the conjugation involves cleavable covalent chemical bonds. This allows for control in terms of the time course over which the polymer may be cleaved from the hedgehog. This covalent bond between the hedgehog protein drug and the polymer may be cleaved by chemical or enzymatic reaction. The polymer-hedgehog protein product retains an acceptable amount of activity. Concurrently, portions of polyethylene glycol are present in the conjugating polymer to endow the polymer-hedgehog protein conjugate with high aqueous solubility and prolonged blood circulation capability. As a result of these improved characteristics the invention contemplates parenteral, aerosol, and oral delivery of both the active polymer-hedgehog protein species and, following hydrolytic cleavage, bioavailability of the hedgehog protein per se, in in vivo applications.

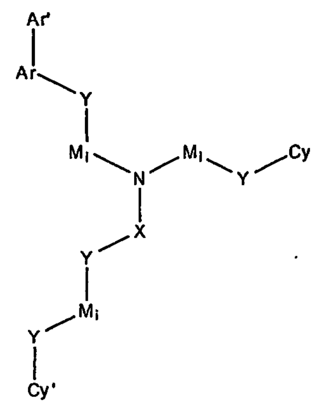
It is to be understood that the reaction schemes described herein are provided for the purposes of illustration only and are not to be limiting with respect to the reactions and structures which may be utilized in the modification of the hedgehog protein, e.g., to achieve solubility, stabilization, and cell membrane affinity for parenteral and oral administration. Generally speaking, the concentrations of reagents used are not critical to carrying out the procedures provided hererin except that the molar amount of activated polymer should be at least equal to, and preferably in excess of, the molar amount of the reactive group (e.g., thiol) on the hedgehog amino acid(s). The reaction of the polymer with the hedgehog to obtain the most preferred conjugated products is readily carried out using a wide variety of reaction schemes. The activity and stability of

the hedgehog protein conjugates can be varied in several ways, by using a polymer of different molecular size. Solubilities of the conjugates can be varied by changing the proportion and size of the polyethylene glycol fragment incorporated in the polymer composition.

3. Small Molecule Agonists

In other embodiments, a hedgehog agonist may be a small organic molecule. Such a small organic molecule may agonize hedgehog signal transduction via an interaction with but not limited to *hedgehog, patched* (*ptc*), *gli*, and/or *smoothened*. It is, therefore, specifically contemplated that these small molecules which enhance or potentiate aspects of *hedgehog*, *ptc*, or *smoothened* signal transduction will likewise be capable of enhancing angiogenesis (or other biological consequences) in normal cells and/or mutant cells. Thus, it is contemplated that in certain embodiments, these compounds may be useful for enhancing or potentiating *hedgehog* activity. In other embodiments, these compounds may be useful for inhibitng *hedgehog* activity in abnormal cells. In preferred embodiments, the subject agonists are organic molecules having a molecular weight less than 2500 amu, more preferably less than 1500 amu, and even more preferably less than 750 amu, and are capable of agonizing hedgehog signaling, preferably specifically in target cells.

For example, agonist compounds useful in the subject methods include compounds represented by general formula (XII):



Formula XII

wherein, as valence and stability permit,

Ar and Ar' independently represent substituted or unsubstituted aryl or heteroaryl rings;

Y, independently for each occurrence, may be absent or represent -N(R)-, -O-, -S-, or -Se-;

X can be selected from -C(=O)-, -C(=S)-, -S(O_2)-, -S(O)-, -C(=NCN)-, -P(=O)(OR_2)-, and a methylene group optionally substituted with 1-2 groups such as lower alkyl, alkenyl, or alkynyl groups;

M represents, independently for each occurrence, a substituted or unsubstituted methylene group, such as -CH_2-, -CHF-, -CHOH-, -CH(Me)-, -C(=O)-, etc., or two M taken together represent substituted or unsubstituted ethene or ethyne;

R represents, independently for each occurrence, H or substituted or unsubstituted aryl, heterocyclyl, heteroaryl, aralkyl, heteroaralkyl, alkynyl, alkenyl, or alkyl, or two R taken together may form a 4- to 8-membered ring, e.g., with N;

Cy and Cy' independenly represent substituted or unsubstituted aryl, heterocyclyl, heteroaryl, or cycloalkyl, including polycyclic groups;

i represents, independently for each occurrence, an integer from 0 to 5, preferably from 0 to 2; and

n, individually for each occurence, represents an integer from 0 to 10, preferably from 0 to 5.

In certain embodiments, M represents, independently for each occurrence, a substituted or unsubstituted methylene group, such as $-CH_2-$, -CHF-, -CHOH-, -CH(Me)-, -C(=O)-, etc.

In certain embodiments, Ar and Ar' represent phenyl rings, e.g., unsubstituted or substituted with one or more groups including heteroatoms such as O, N, and S. In certain embodiments, at least one of Ar and Ar' represents a phenyl ring. In certain embodiments, at least one of Ar and Ar' represents a heteroaryl ring, e.g., a pyridyl, thiazolyl, thienyl, pyrimidyl, etc. In certain embodiments, Y and Ar' are attached to Ar in a meta and/or 1,3-relationship.

In certain embodiments, Y is absent from all positions. In embodiments wherein Y is present in a position, i preferably represents an integer from 1-2 in an adjacent M_i if i=0 would result in two occurrences of Y being directly attached, or an occurrence of Y being directly attached to N.

In certain embodiments, Cy' is a substituted or unsubstituted aryl or heteroaryl. In certain embodiments, Cy' is directly attached to X. In certain embodiments, Cy' is a substituted or unsubstituted bicyclic or heteroaryl ring, preferably both bicyclic and heteroaryl, such as benzothiophene, benzofuran, benzopyrrole, benzopyridine, etc. In certain embodiments, Cy' is a monocyclic aryl or heteroaryl ring substituted at least with a substituted or unsubstituted aryl or heteroaryl ring, e.g., forming a biaryl system. In certain embodiments, Cy' includes two substituted or unsubstituted aryl or heteroaryl rings, e.g., the same or different, directly connected by one or more bonds, e.g., to form a biaryl or bicyclic ring system.

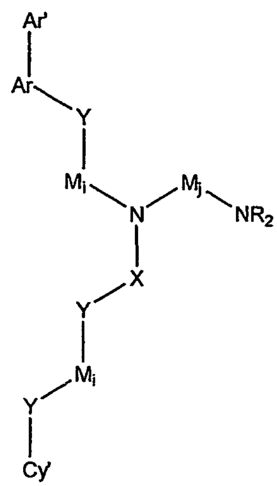
In certain embodiments, X is selected from -C(=O)-, -C(=S)-, and $-S(O_2)-$.

In certain embodiments, Cy represents a substituted or unsubstituted non-aromatic carbocyclic or heterocyclic ring, i.e., including at least one sp^3 hybridized atom, and preferably a plurality of sp^3 hybridized atoms. In certain embodiments, Cy includes an amine within the atoms of the ring or on a substitutent of the ring, e.g., Cy is

pyridyl, imidazolyl, pyrrolyl, piperidyl, pyrrolidyl, piperazyl, etc., and/or bears an amino substituent. . In certain embodiments, Cy is a 5- to 7-membered ring. In certain embodiments, Cy is directly attached to N. In embodiments wherein Cy is a six-membered ring directly attached to N and bears an amino substituent at the 4 position of the ring relative to N, the N and amine substituents may be disposed *trans* on the ring.

In certain embodiments, substituents on Ar or Ar' are selected from halogen, lower alkyl, lower alkenyl, aryl, heteroaryl, carbonyl, thiocarbonyl, ketone, aldehyde, amino, acylamino, cyano, nitro, hydroxyl, azido, sulfonyl, sulfoxido, sulfate, sulfonate, sulfamoyl, sulfonamido, phosphoryl, phosphonate, phosphinate, $-(CH_2)_p$alkyl, $-(CH_2)_p$alkenyl, $-(CH_2)_p$alkynyl, $-(CH_2)_p$aryl, $-(CH_2)_p$aralkyl, $-(CH_2)_pOH$, $-(CH_2)_pO$-lower alkyl, $-(CH_2)_pO$-lower alkenyl, $-O(CH_2)_nR$, $-(CH_2)_pSH$, $-(CH_2)_pS$-lower alkyl, $-(CH_2)_pS$-lower alkenyl, $-S(CH_2)_nR$, $-(CH_2)_pN(R)_2$, $-(CH_2)_pNR$-lower alkyl, $-(CH_2)_pNR$-lower alkenyl, $-NR(CH_2)_nR$, and protected forms of the above, wherein p, individually for each occurence, represents an integer from 0 to 10, preferably from 0 to 5.

In certain embodiments, compounds useful in the present invention may be represented by general formula (XIII):



Formula XIII

wherein, as valence and stability permit,

Ar and Ar' independently represent substituted or unsubstituted aryl or heteroaryl rings;

Y, independently for each occurrence, may be absent or represent -N(R)-, -O-, -S-, or -Se-;

X can be selected from -C(=O)-, -C(=S)-, $-S(O_2)-$, -S(O)-, -C(=NCN)-, $-P(=O)(OR_2)-$, and a methylene group optionally substituted with 1-2 groups such as lower alkyl, alkenyl, or alkynyl groups;

M represents, independently for each occurrence, a substituted or unsubstituted methylene group, such as $-CH_2-$, -CHF-, -CHOH-, -CH(Me)-, -C(=O)-, etc., or two M taken together represent substituted or unsubstituted ethene or ethyne, wherein some or all occurrences of M in M_j form all or part of a cyclic structure;

R represents, independently for each occurrence, H or substituted or unsubstituted aryl, heterocyclyl, heteroaryl, aralkyl, heteroaralkyl, alkynyl, alkenyl, or alkyl, or two R taken together may form a 4- to 8-membered ring, e.g., with N;

Cy' represents a substituted or unsubstituted aryl, heterocyclyl, heteroaryl, or cycloalkyl, including polycyclic groups;

j represents, independently for each occurrence, an integer from 0 to 10, preferably from 2 to 7;

i represents, independently for each occurrence, an integer from 0 to 5, preferably from 0 to 2; and

n, individually for each occurence, represents an integer from 0 to 10, preferably from 0 to 5.

In certain embodiments, M represents, independently for each occurrence, a substituted or unsubstituted methylene group, such as $-CH_2-$, -CHF-, -CHOH-, -CH(Me)-, -C(=O)-, etc.

In certain embodiments, Ar and Ar' represent phenyl rings, e.g., unsubstituted or substituted with one or more groups including heteroatoms such as O, N, and S. In certain embodiments, at least one of Ar and Ar' represents a phenyl ring. In certain embodiments, at least one of Ar and Ar' represents a heteroaryl ring, e.g., a pyridyl, thiazolyl, thienyl, pyrimidyl, etc. In certain embodiments, Y and Ar' are attached to Ar in a meta and/or 1,3-relationship.

In certain embodiments, Y is absent from all positions. In embodiments wherein Y is present in a position, i preferably represents an integer from 1-2 in an adjacent M_i if i=0 would result in two occurrences of Y being directly attached, or an occurrence of Y being directly attached to N or NR_2.

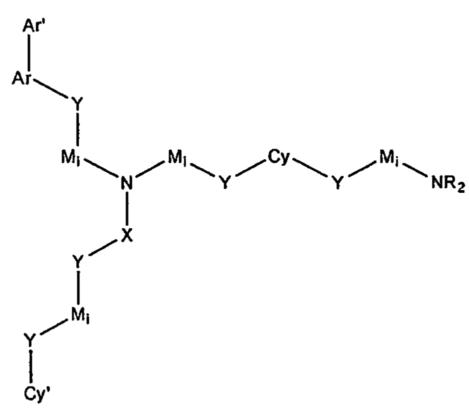
In certain embodiments, Cy' is a substituted or unsubstituted aryl or heteroaryl. In certain embodiments, Cy' is directly attached to X. In certain embodiments, Cy' is a substituted or unsubstituted bicyclic or heteroaryl ring, preferably both bicyclic and heteroaryl, such as benzothiophene, benzofuran, benzopyrrole, benzopyridine, etc. In certain embodiments, Cy' is a monocyclic aryl or heteroaryl ring substituted at least with a substituted or unsubstituted aryl or heteroaryl ring, e.g., forming a biaryl system. In certain embodiments, Cy' includes two substituted or unsubstituted aryl or heteroaryl rings, e.g., the same or different, directly connected by one or more bonds, e.g., to form a biaryl or bicyclic ring system.

In certain embodiments, X is selected from -C(=O)-, -C(=S)-, and $-S(O_2)-$.

In certain embodiments, NR_2 represents a primary amine or a secondary or tertiary amine substituted with one or two lower alkyl groups, aryl groups, or aralkyl groups, respectively, preferably a primary amine.

In certain embodiments, substituents on Ar or Ar' are selected from halogen, lower alkyl, lower alkenyl, aryl, heteroaryl, carbonyl, thiocarbonyl, ketone, aldehyde, amino, acylamino, cyano, nitro, hydroxyl, azido, sulfonyl, sulfoxido, sulfate, sulfonate, sulfamoyl, sulfonamido, phosphoryl, phosphonate, phosphinate, $-(CH_2)_p$alkyl, $-(CH_2)_p$alkenyl, $-(CH_2)_p$alkynyl, $-(CH_2)_p$aryl, $-(CH_2)_p$aralkyl, $-(CH_2)_pOH$, $-(CH_2)_pO$-lower alkyl, $-(CH_2)_pO$-lower alkenyl, $-O(CH_2)_nR$, $-(CH_2)_pSH$, $-(CH_2)_pS$-lower alkyl, $-(CH_2)_pS$-lower alkenyl, $-S(CH_2)_nR$, $-(CH_2)_pN(R)_2$, $-(CH_2)_pNR$-lower alkyl, $-(CH_2)_pNR$-lower alkenyl, $-NR(CH_2)_nR$, and protected forms of the above, wherein p, individually for each occurence, represents an integer from 0 to 10, preferably from 0 to 5.

In certain embodiments, compounds useful in the present invention may be represented by general formula (XIV):



Formula XIV

wherein, as valence and stability permit,

Ar and Ar' independently represent substituted or unsubstituted aryl or heteroaryl rings;

Y, independently for each occurrence, may be absent or represent -N(R)-, -O-, -S-, or -Se-;

X can be selected from -C(=O)-, -C(=S)-, -$S(O_2)$-, -S(O)-, -C(=NCN)-, -P(=O)(OR_2)-, and a methylene group optionally substituted with 1-2 groups such as lower alkyl, alkenyl, or alkynyl groups;

M represents, independently for each occurrence, a substituted or unsubstituted methylene group, such as -CH_2-, -CHF-, -CHOH-, -CH(Me)-, -C(=O)-, etc., or two M taken together represent substituted or unsubstituted ethene or ethyne;

R represents, independently for each occurrence, H or substituted or unsubstituted aryl, heterocyclyl, heteroaryl, aralkyl, heteroaralkyl, alkynyl, alkenyl, or alkyl, or two R taken together may form a 4- to 8-membered ring, e.g., with N;

Cy and Cy' independenly represent substituted or unsubstituted aryl, heterocyclyl, heteroaryl, or cycloalkyl, including polycyclic groups;

i represents, independently for each occurrence, an integer from 0 to 5, preferably from 0 to 2; and

n, individually for each occurence, represents an integer from 0 to 10, preferably from 0 to 5.

In certain embodiments, M represents, independently for each occurrence, a substituted or unsubstituted methylene group, such as -CH_2-, -CHF-, -CHOH-, -CH(Me)-, -C(=O)-, etc.

In certain embodiments, Ar and Ar' represent phenyl rings, e.g., unsubstituted or substituted with one or more groups including heteroatoms such as O, N, and S. In certain embodiments, at least one of Ar and Ar' represents a phenyl ring. In certain embodiments, at least one of Ar and Ar' represents a heteroaryl ring, e.g., a pyridyl, thiazolyl, thienyl, pyrimidyl, etc. In certain embodiments, Y and Ar' are attached to Ar in a meta and/or 1,3-relationship.

In certain embodiments, Y is absent from all positions. In embodiments wherein Y is present in a position, i preferably represents an integer from 1-2 in an adjacent M_i if

i=0 would result in two occurrences of Y being directly attached, or an occurrence of Y being directly attached to N or NR_2.

In certain embodiments, Cy' is a substituted or unsubstituted aryl or heteroaryl. In certain embodiments, Cy' is directly attached to X. In certain embodiments, Cy' is a substituted or unsubstituted bicyclic or heteroaryl ring, preferably both bicyclic and heteroaryl, such as benzothiophene, benzofuran, benzopyrrole, benzopyridine, etc. In certain embodiments, Cy' is a monocyclic aryl or heteroaryl ring substituted at least with a substituted or unsubstituted aryl or heteroaryl ring, e.g., forming a biaryl system. In certain embodiments, Cy' includes two substituted or unsubstituted aryl or heteroaryl rings, e.g., the same or different, directly connected by one or more bonds, e.g., to form a biaryl or bicyclic ring system.

In certain embodiments, X is selected from -C(=O)-, -C(=S)-, and $-S(O_2)-$.

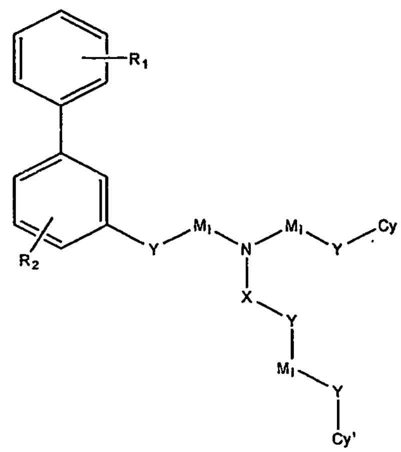
In certain embodiments, NR_2 represents a primary amine or a secondary or tertiary amine substituted with one or two lower alkyl groups, aryl groups, or aralkyl groups, respectively, preferably a primary amine.

In certain embodiments, Cy represents a substituted or unsubstituted non-aromatic carbocyclic or heterocyclic ring, i.e., including at least one sp^3 hybridized atom, and preferably a plurality of sp^3 hybridized atoms. In certain embodiments, Cy is directly attached to N and/or to NR_2. In certain embodiments, Cy is a 5- to 7-membered ring. In embodiments wherein Cy is a six-membered ring directly attached to N and bears an amino substituent at the 4 position of the ring relative to N, the N and amine substituents may be disposed *trans* on the ring.

In certain embodiments, substituents on Ar or Ar' are selected from halogen, lower alkyl, lower alkenyl, aryl, heteroaryl, carbonyl, thiocarbonyl, ketone, aldehyde, amino, acylamino, cyano, nitro, hydroxyl, azido, sulfonyl, sulfoxido, sulfate, sulfonate, sulfamoyl, sulfonamido, phosphoryl, phosphonate, phosphinate, $-(CH_2)_p$alkyl, $-(CH_2)_p$alkenyl, $-(CH_2)_p$alkynyl, $-(CH_2)_p$aryl, $-(CH_2)_p$aralkyl, $-(CH_2)_pOH$, $-(CH_2)_pO$-lower alkyl, $-(CH_2)_pO$-lower alkenyl, $-O(CH_2)_nR$, $-(CH_2)_pSH$, $-(CH_2)_pS$-lower alkyl, $-(CH_2)_pS$-lower alkenyl, $-S(CH_2)_nR$, $-(CH_2)_pN(R)_2$, $-(CH_2)_pNR$-lower alkyl, $-(CH_2)_pNR$-lower alkenyl, $-NR(CH_2)_nR$, and protected forms of the above, wherein p,

individually for each occurence, represents an integer from 0 to 10, preferably from 0 to 5.

In certain embodiments, compounds useful in the subject methods include compounds represented by general forumla (XV):



Formula XV

wherein, as valence and stability permit,

Cy' represents a substituted or unsubstituted aryl or heteroaryl ring, including polycyclics;

Y, independently for each occurrence, may be absent or represent -N(R)-, -O-, -S-, or -Se-;

X can be selected from -C(=O)-, -C(=S)-, $-S(O_2)-$, -S(O)-, -C(=NCN)-, $-P(=O)(OR_2)-$, and a methylene group optionally substituted with 1-2 groups such as lower alkyl, alkenyl, or alkynyl groups;

M represents, independently for each occurrence, a substituted or unsubstituted methylene group, such as $-CH_2-$, -CHF-, -CHOH-, -CH(Me)-, -C(=O)-, etc., or two M taken together represent substituted or unsubstituted ethene or ethyne;

R represents, independently for each occurrence, H or substituted or unsubstituted aryl, heterocyclyl, heteroaryl, aralkyl, heteroaralkyl, alkynyl, alkenyl, or alkyl, or two R taken together may form a 4- to 8-membered ring, e.g., with N;

R_1 and R_2 represent, independently and as valency permits, from 0-5 substituents on the ring to which it is attached, selected from halogen, lower alkyl, lower alkenyl, aryl, heteroaryl, carbonyl, thiocarbonyl, ketone, aldehyde, amino, acylamino, amido, amidino, cyano, nitro, hydroxyl, azido, sulfonyl, sulfoxido, sulfate, sulfonate, sulfamoyl, sulfonamido, phosphoryl, phosphonate, phosphinate, $-(CH_2)_p$alkyl, $-(CH_2)_p$alkenyl, $-(CH_2)_p$alkynyl, $-(CH_2)_p$aryl, $-(CH_2)_p$aralkyl, $-(CH_2)_pOH$, $-(CH_2)_pO$-lower alkyl, $-(CH_2)_pO$-lower alkenyl, $-O(CH_2)_nR$, $-(CH_2)_pSH$, $-(CH_2)_pS$-lower alkyl, $-(CH_2)_pS$-lower alkenyl, $-S(CH_2)_nR$, $-(CH_2)_pN(R)_2$, $-(CH_2)_pNR$-lower alkyl, $-(CH_2)_pNR$-lower alkenyl, $-NR(CH_2)_nR$, and protected forms of the above;

Cy represents substituted or unsubstituted aryl, heterocyclyl, heteroaryl, or cycloalkyl, including polycyclic groups;

i represents, independently for each occurrence, an integer from 0 to 5, preferably from 0 to 2; and

p and n, individually for each occurrence, represent integers from 0 to 10, preferably from 0 to 5.

In certain embodiments, M represents, independently for each occurrence, a substituted or unsubstituted methylene group, such as $-CH_2-$, -CHF-, -CHOH-, -CH(Me)-, -C(=O)-, etc.

In certain embodiments, Cy' represents a substituted or unsubstituted bicyclic or heterocyclic ring system, preferably both bicyclic and heteroaryl, such as benzothiophene, benzofuran, benzopyrrole, benzopyridine, etc. In certain embodiments, Cy' is directly attached to X. In certain embodiments, Cy' is a monocyclic aryl or heteroaryl ring substituted at least with a substituted or unsubstituted aryl or heteroaryl ring, e.g., forming a biaryl system. In certain embodiments, Cy' includes two substituted or unsubstituted aryl or heteroaryl rings, e.g., the same or different, directly connected by one or more bonds, e.g., to form a biaryl or bicyclic ring system.

In certain embodiments, Y is absent from all positions. In embodiments wherein Y is present in a position, i preferably represents an integer from 1-2 in an adjacent M_i if

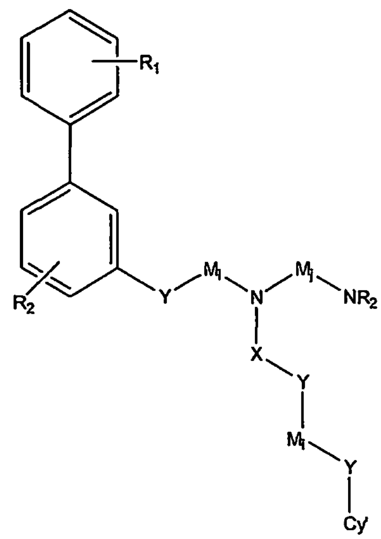
i=0 would result in two occurrences of Y being directly attached, or an occurrence of Y being directly attached to N.

In certain embodiments, X is selected from -C(=O)-, -C(=S)-, and $-S(O_2)-$.

In certain embodiments, Cy represents a substituted or unsubstituted non-aromatic carbocyclic or heterocyclic ring, i.e., including at least one sp^3 hybridized atom, and preferably a plurality of sp^3 hybridized atoms. In certain embodiments, Cy includes an amine within the atoms of the ring or on a substitutent of the ring, e.g., Cy is pyridyl, imidazolyl, pyrrolyl, piperidyl, pyrrolidyl, piperazyl, etc., and/or bears an amino substituent. In certain embodiments, Cy is directly attached to N. In certain embodiments, Cy is a 5- to 7-membered ring. In embodiments wherein Cy is a six-membered ring directly attached to N and bears an amino substituent at the 4 position of the ring relative to N, the N and amine substituents may be disposed *trans* on the ring.

In certain embodiments, R_1 and R_2 represent, independently and as valency permits, from 0-5 substituents on the ring to which it is attached, selected from halogen, lower alkyl, lower alkenyl, carbonyl, thiocarbonyl, ketone, aldehyde, amino, acylamino, cyano, nitro, hydroxyl, azido, sulfonyl, sulfoxido, sulfate, sulfonate, sulfamoyl, sulfonamido, phosphoryl, phosphonate, phosphinate, $-(CH_2)_p$alkyl, $-(CH_2)_p$alkenyl, $-(CH_2)_p$alkynyl, $-(CH_2)_p$aryl, $-(CH_2)_p$aralkyl, $-(CH_2)_pOH$, $-(CH_2)_pO$-lower alkyl, $-(CH_2)_pO$-lower alkenyl, $-O(CH_2)_nR$, $-(CH_2)_pSH$, $-(CH_2)_pS$-lower alkyl, $-(CH_2)_pS$-lower alkenyl, $-S(CH_2)_nR$, $-(CH_2)_pN(R)_2$, $-(CH_2)_pNR$-lower alkyl, $-(CH_2)_pNR$-lower alkenyl, $-NR(CH_2)_nR$, and protected forms of the above, wherein p, individually for each occurence, represents an integer from 0 to 10, preferably from 0 to 5.

In certain embodiments, compounds useful in the present invention may be represented by general formula (XVI):



Formula XVI

wherein, as valence and stability permit,

Cy' represents a substituted or unsubstituted aryl or heteroaryl ring, including polycyclics;

Y, independently for each occurrence, may be absent or represent -N(R)-, -O-, -S-, or -Se-;

X can be selected from -C(=O)-, -C(=S)-, -S(O_2)-, -S(O)-, -C(=NCN)-, -P(=O)(OR_2)-, and a methylene group optionally substituted with 1-2 groups such as lower alkyl, alkenyl, or alkynyl groups;

M represents, independently for each occurrence, a substituted or unsubstituted methylene group, such as -CH_2-, -CHF-, -CHOH-, -CH(Me)-, -C(=O)-, etc., or two M taken together represent substituted or unsubstituted ethene or ethyne;

R represents, independently for each occurrence, H or substituted or unsubstituted aryl, heterocyclyl, heteroaryl, aralkyl, heteroaralkyl, alkynyl, alkenyl, or alkyl, or two R taken together may form a 4- to 8-membered ring, e.g., with N;

R_1 and R_2 represent, independently and as valency permits, from 0-5 substituents on the ring to which it is attached, selected from halogen, lower alkyl, lower alkenyl, aryl, heteroaryl, carbonyl, thiocarbonyl, ketone, aldehyde, amino, acylamino, amido, amidino, cyano, nitro, hydroxyl, azido, sulfonyl, sulfoxido, sulfate, sulfonate, sulfamoyl,

sulfonamido, phosphoryl, phosphonate, phosphinate, -$(CH_2)_p$alkyl, -$(CH_2)_p$alkenyl, -$(CH_2)_p$alkynyl, -$(CH_2)_p$aryl, -$(CH_2)_p$aralkyl, -$(CH_2)_p$OH, -$(CH_2)_p$O-lower alkyl, -$(CH_2)_p$O-lower alkenyl, -O$(CH_2)_n$R, -$(CH_2)_p$SH, -$(CH_2)_p$S-lower alkyl, -$(CH_2)_p$S-lower alkenyl, -S$(CH_2)_n$R, -$(CH_2)_p$N(R)$_2$, -$(CH_2)_p$NR-lower alkyl, -$(CH_2)_p$NR-lower alkenyl, -NR$(CH_2)_n$R, and protected forms of the above;

Cy' representsa substituted or unsubstituted aryl, heterocyclyl, heteroaryl, or cycloalkyl, including polycyclic groups;

j represents, independently for each occurrence, an integer from 0 to 10, preferably from 2 to 7;

i represents, independently for each occurrence, an integer from 0 to 5, preferably from 0 to 2; and

p and n, individually for each occurrence, represent integers from 0 to 10, preferably from 0 to 5.

In certain embodiments, M represents, independently for each occurrence, a substituted or unsubstituted methylene group, such as -CH_2-, -CHF-, -CHOH-, -CH(Me)-, -C(=O)-, etc.

In certain embodiments, Cy' represents a substituted or unsubstituted bicyclic or heterocyclic ring system, preferably both bicyclic and heteroaryl, such as benzothiophene, benzofuran, benzopyrrole, benzopyridine, etc. In certain embodiments, Cy' is directly attached to X. In certain embodiments, Cy' is a monocyclic aryl or heteroaryl ring substituted at least with a substituted or unsubstituted aryl or heteroaryl ring, e.g., forming a biaryl system. In certain embodiments, Cy' includes two substituted or unsubstituted aryl or heteroaryl rings, e.g., the same or different, directly connected by one or more bonds, e.g., to form a biaryl or bicyclic ring system.

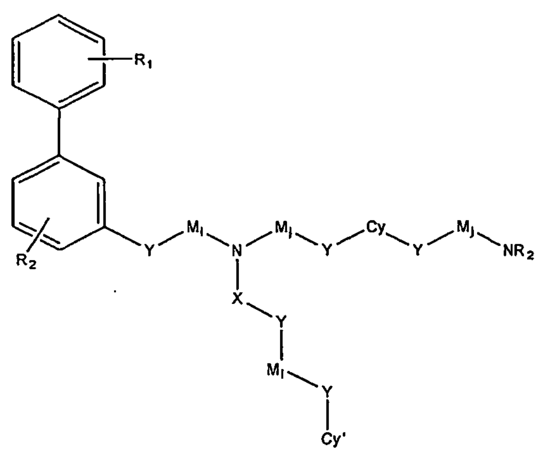
In certain embodiments, Y is absent from all positions. In embodiments wherein Y is present in a position, i preferably represents an integer from 1-2 in an adjacent M_i if i=0 would result in two occurrences of Y being directly attached, or an occurrence of Y being directly attached to N or NR_2.

In certain embodiments, X is selected from -C(=O)-, -C(=S)-, and -S(O_2)-.

In certain embodiments, NR_2 represents a primary amine or a secondary or tertiary amine substituted with one or two lower alkyl groups, aryl groups, or aralkyl groups, respectively, preferably a primary amine.

In certain embodiments, R_1 and R_2 represent, independently and as valency permits, from 0-5 substituents on the ring to which it is attached, selected from halogen, lower alkyl, lower alkenyl, carbonyl, thiocarbonyl, ketone, aldehyde, amino, acylamino, cyano, nitro, hydroxyl, azido, sulfonyl, sulfoxido, sulfate, sulfonate, sulfamoyl, sulfonamido, phosphoryl, phosphonate, phosphinate, $-(CH_2)_p$alkyl, $-(CH_2)_p$alkenyl, $-(CH_2)_p$alkynyl, $-(CH_2)_p$aryl, $-(CH_2)_p$aralkyl, $-(CH_2)_pOH$, $-(CH_2)_pO$-lower alkyl, $-(CH_2)_pO$-lower alkenyl, $-O(CH_2)_nR$, $-(CH_2)_pSH$, $-(CH_2)_pS$-lower alkyl, $-(CH_2)_pS$-lower alkenyl, $-S(CH_2)_nR$, $-(CH_2)_pN(R)_2$, $-(CH_2)_pNR$-lower alkyl, $-(CH_2)_pNR$-lower alkenyl, $-NR(CH_2)_nR$, and protected forms of the above, wherein p, individually for each occurence, represents an integer from 0 to 10, preferably from 0 to 5.

In certain embodiments, compounds useful in the present invention may be represented by general formula (XVII):



Formula XVII

wherein, as valence and stability permit,

Cy' represents a substituted or unsubstituted aryl or heteroaryl ring, including polycyclics;

Y, independently for each occurrence, may be absent or represent -N(R)-, -O-, -S-, or -Se-;

X can be selected from -C(=O)-, -C(=S)-, -S(O_2)-, -S(O)-, -C(=NCN)-, -P(=O)(OR_2)-, and a methylene group optionally substituted with 1-2 groups such as lower alkyl, alkenyl, or alkynyl groups;

M represents, independently for each occurrence, a substituted or unsubstituted methylene group, such as -CH_2-, -CHF-, -CHOH-, -CH(Me)-, -C(=O)-, etc., or two M taken together represent substituted or unsubstituted ethene or ethyne;

R represents, independently for each occurrence, H or substituted or unsubstituted aryl, heterocyclyl, heteroaryl, aralkyl, heteroaralkyl, alkynyl, alkenyl, or alkyl, or two R taken together may form a 4- to 8-membered ring, e.g., with N;

Cy represents substituted or unsubstituted aryl, heterocyclyl, heteroaryl, or cycloalkyl, including polycyclic groups;

i represents, independently for each occurrence, an integer from 0 to 5, preferably from 0 to 2; and

n and p, individually for each occurrence, represent integers from 0 to 10, preferably from 0 to 5.

In certain embodiments, M represents, independently for each occurrence, a substituted or unsubstituted methylene group, such as -CH_2-, -CHF-, -CHOH-, -CH(Me)-, -C(=O)-, etc.

In certain embodiments, Cy' represents a substituted or unsubstituted bicyclic or heteroaryl ring system, preferably both bicyclic and heteroaryl, e.g., benzothiophene, benzofuran, benzopyrrole, benzopyridyl, etc. In certain embodiments, Cy' is directly attached to X. In certain embodiments, Cy' is a monocyclic aryl or heteroaryl ring substituted at least with a substituted or unsubstituted aryl or heteroaryl ring, e.g., forming a biaryl system. In certain embodiments, Cy' includes two substituted or unsubstituted aryl or heteroaryl rings, e.g., the same or different, directly connected by one or more bonds, e.g., to form a biaryl or bicyclic ring system.

In certain embodiments, Y is absent from all positions. In embodiments wherein Y is present in a position, i preferably represents an integer from 1-2 in an adjacent M_i if i=0 would result in two occurrences of Y being directly attached, or an occurrence of Y being directly attached to N or NR_2.

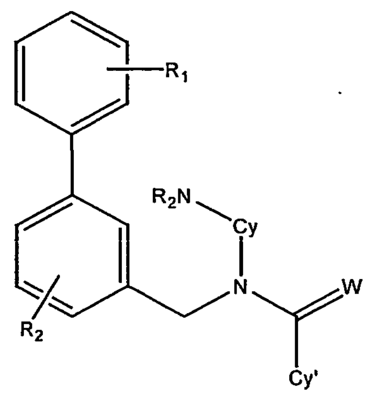
In certain embodiments, X is selected from -C(=O)-, -C(=S)-, and $-S(O_2)-$.

In certain embodiments, NR_2 represents a primary amine or a secondary or tertiary amine substituted with one or two lower alkyl groups, aryl groups, or aralkyl groups, respectively, preferably a primary amine.

In certain embodiments, Cy represents a substituted or unsubstituted non-aromatic carbocyclic or heterocyclic ring, i.e., including at least one sp^3 hybridized atom, and preferably a plurality of sp^3 hybridized atoms. In certain embodiments, Cy is directly attached to N and/or to NR_2. In certain embodiments, Cy is a 5- to 7-membered ring. In embodiments wherein Cy is a six-membered ring directly attached to N and bears an amino substituent at the 4 position of the ring relative to N, the N and amine substituents may be disposed *trans* on the ring.

In certain embodiments, R_1 and R_2 represent, independently and as valency permits, from 0-5 substituents on the ring to which it is attached, selected from halogen, lower alkyl, lower alkenyl, carbonyl, thiocarbonyl, ketone, aldehyde, amino, acylamino, cyano, nitro, hydroxyl, azido, sulfonyl, sulfoxido, sulfate, sulfonate, sulfamoyl, sulfonamido, phosphoryl, phosphonate, phosphinate, $-(CH_2)_p$alkyl, $-(CH_2)_p$alkenyl, $-(CH_2)_p$alkynyl, $-(CH_2)_p$aryl, $-(CH_2)_p$aralkyl, $-(CH_2)_pOH$, $-(CH_2)_pO$-lower alkyl, $-(CH_2)_pO$-lower alkenyl, $-O(CH_2)_nR$, $-(CH_2)_pSH$, $-(CH_2)_pS$-lower alkyl, $-(CH_2)_pS$-lower alkenyl, $-S(CH_2)_nR$, $-(CH_2)_pN(R)_2$, $-(CH_2)_pNR$-lower alkyl, $-(CH_2)_pNR$-lower alkenyl, $-NR(CH_2)_nR$, and protected forms of the above, wherein p, individually for each occurence, represents an integer from 0 to 10, preferably from 0 to 5.

In certain embodiments, a subject compound has the structure of Formula XVIII:



wherein, as valence and stability permit,

Cy represents a substituted or unsubstituted heterocyclyl or cycloalkyl;

Cy' is a substituted or unsubstituted aryl or heteroaryl ring;

W is O or S;

R represents, independently for each occurrence, H or substituted or unsubstituted aryl, heterocyclyl, heteroaryl, aralkyl, heteroaralkyl, alkynyl, alkenyl, or alkyl, or two R taken together may form a 4- to 8-membered ring, e.g., with N;

R_1 and R_2 represent, independently and as valency permits, from 0-5 substituents on the ring to which it is attached, selected from halogen, lower alkyl, lower alkenyl, aryl, heteroaryl, carbonyl, thiocarbonyl, ketone, aldehyde, amino, acylamino, amido, amidino, cyano, nitro, hydroxyl, azido, sulfonyl, sulfoxido, sulfate, sulfonate, sulfamoyl, sulfonamido, phosphoryl, phosphonate, phosphinate, $-(CH_2)_p$alkyl, $-(CH_2)_p$alkenyl, $-(CH_2)_p$alkynyl, $-(CH_2)_p$aryl, $-(CH_2)_p$aralkyl, $-(CH_2)_pOH$, $-(CH_2)_pO$-lower alkyl, $-(CH_2)_pO$-lower alkenyl, $-O(CH_2)_nR$, $-(CH_2)_pSH$, $-(CH_2)_pS$-lower alkyl, $-(CH_2)_pS$-lower alkenyl, $-S(CH_2)_nR$, $-(CH_2)_pN(R)_2$, $-(CH_2)_pNR$-lower alkyl, $-(CH_2)_pNR$-lower alkenyl, $-NR(CH_2)_nR$, and protected forms of the above;

n and p, individually for each occurrence, represent integers from 0 to 10.

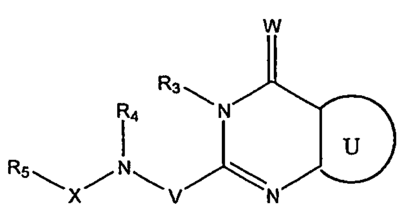
In certain embodiments, Cy' represents a substituted or unsubstituted bicyclic or heteroaryl ring system, preferably both bicyclic and heteroaryl, e.g., benzothiophene, benzofuran, benzopyrrole, benzopyridyl, etc. In certain embodiments, Cy' is directly attached to X.

In certain embodiments, NR_2 represents a primary amine or a secondary or tertiary amine substituted with one or two lower alkyl groups, aryl groups, or aralkyl groups, respectively, preferably a primary amine.

In certain embodiments, Cy represents a substituted or unsubstituted saturated carbocyclic or heterocyclic ring, i.e., composed of a plurality of sp^3 hybridized atoms. In certain embodiments, Cy is a 5- to 7-membered ring. In embodiments wherein Cy is a six-membered ring directly attached to N and bears an amino substituent at the 4 position of the ring relative to N, the N and amine substituents may be disposed *trans* on the ring.

In certain embodiments, R_1 and R_2 represent, independently and as valency permits, from 0-5 substituents on the ring to which it is attached, selected from halogen, lower alkyl, lower alkenyl, carbonyl, thiocarbonyl, ketone, aldehyde, amino, acylamino, cyano, nitro, hydroxyl, sulfonyl, sulfoxido, sulfate, sulfonate, sulfamoyl, sulfonamido, -$(CH_2)_p$alkyl, -$(CH_2)_p$alkenyl, -$(CH_2)_p$alkynyl, -$(CH_2)_p$aryl, -$(CH_2)_p$aralkyl, -$(CH_2)_pOH$, -$(CH_2)_pO$-lower alkyl, -$(CH_2)_pO$-lower alkenyl, -$O(CH_2)_nR$, -$(CH_2)_pSH$, -$(CH_2)_pS$-lower alkyl, -$(CH_2)_pS$-lower alkenyl, -$S(CH_2)_nR$, -$(CH_2)_pN(R)_2$, -$(CH_2)_pNR$-lower alkyl, -$(CH_2)_pNR$-lower alkenyl, -$NR(CH_2)_nR$, and protected forms of the above.

In certain embodiments, a subject compound has a structure of Formula XIX:



wherein, as valence and stability permit,

U represents a substituted or unsubstituted aryl or heteroaryl ring fused to the nitrogen-containing ring;

V represents a lower alkylene group, such as methylene, 1,2-ethylene, 1,1-ethylene, 1,1-propylene, 1,2-propylene, 1,3-propylene, etc.;

W represents S or O, preferably O;

X represents C=O, C=S, or SO_2;

R_3 represents substituted or unsubstituted aryl, heteroaryl, lower alkyl, lower alkenyl, lower alkynyl, carbocyclyl, carbocyclylalkyl, heterocyclyl, heterocyclylalkyl, aralkyl, or heteroaralkyl;

R_4 represents substituted or unsubstituted aralkyl or lower alkyl, such as phenethyl, benzyl, or aminoalkyl, etc.;

R_5 represents substituted or unsubstituted aryl, heteroaryl, aralkyl, or heteroaralkyl, including polycyclic aromatic or heteroaromatic groups.

In certain embodiments, U represents a phenyl ring fused to the nitrogen-containing ring.

In certain embodiments, R_3 is selected from substituted or unsubstituted aryl, heteroaryl, lower alkyl, lower alkenyl, aralkyl, and heteroaralkyl.

In certain embodiments, R_4 is an unsubstituted lower alkyl group, or is a lower alkyl group substituted with a secondary or tertiary amine.

In certain embodiments, R_5 is selected from substituted or unsubstituted phenyl or naphthyl, or is a diarylalkyl group, such as 2,2-diphenylethyl, diphenylmethyl, etc.

Moreover, the subject methods can be performed on cells which are provided in culture (*in vitro*), or on cells in a whole animal (*in vivo*). See, for example, PCT publications WO 95/18856 and WO 96/17924 (the specifications of which are expressly incorporated by reference herein).

VI. Testing for Biological Activity

While many bioassays have been used to demonstrate hedgehog activity, the C3H10T1/2 cell line provides a simple system for assessing hedgehog function without

the complication of having to work with primary cell cultures or organ explants. The mouse embryonic fibroblast line C3HlOT1/2 is a mesenchymal stem cell line that, under defined conditions, can differentiate into adipocytes, chondrocytes, and bone osteoblasts (Taylor, S.M., and Jones, P.A., Cell 17: 771-779 (1979) and Wang, E.A., et al., Growth Factors 9: 57-71 (1993)). Bone morphogenic proteins drive the differentiation of C3H lOT 1/2 cells into the bone cell lineage and alkaline phosphatase induction has been used as a marker for this process (Wang et al., supra). Shh has a similar effect on C3H10T1/2 cells (Kinto, N. et al., FEBS Letts. 404: 319-323 (1997)) and we routinely use the alkaline phosphatase induction by Shh as a quantitative measure of its in vitro potency. Shh treatment also produces a dose-dependent increase in gli-1 and ptc-1 expression, which can be readily detected by a PCR-based analysis.

We found that hedgehog protein can upregulate fibroblast expression of angiogenic growth factors, including VEGF121, VEGF165, VEGF189, Ang-1, and Ang-2 (Example 4). Thus, the procedure outlined in Example 4 provides a new method of measuring the in vitro angiogenic potential of hedgehog. Without wishing to be bound by any particular theory, this upregulation may explain the mechanism whereby hedgehog exerts its angiogenic effect.

Similarly, this cell line provides a simple bioassay to test the agonistic or antagonistic properties of the hedgehog therapeutics of the present invention. In preferred embodiments, agonists would be expected to induce alkaline phosphatase in CSH10T1/2 cells. In other embodiments, antagonists would be expected to inhibit the induction of alkaline phosphatase by exogenous hedgehog.

Further, persons having ordinary skill in the art will recognize means for determining if the hedgehog agents used in the present methods are efficacious in vivo. For instance, clinicians have available to them a variety of non-invasive tests such as echograms, electrocardiograms, CAT scans, MRI to determine vascular and cardiac functioning. Other methods include angiography and other more invasive physiological testing methods. For patients with neuropathies, nerve conduction velocity tests may be routinely performed. To test for the anti-angiogenic function of hedgehog antagonists, persons of ordinary skill in the art way use a variety of imaging methods such as CAT

and MRI scans, as well as more invasive tests to look at blood chemistry and tumor metabolism.

VII. Subjects for Treatment

As a general matter, the methods of the present invention may be utilized for any mammalian subject needing modulation of angiogenesis. Mammalian subjects which may be treated according to the methods of the invention include, but are not limited to, human subjects or patients. In addition, however, the invention may be employed in the treatment of domesticated mammals which are maintained as human companions (e.g., dogs, cats, horses), which have significant commercial value (e.g., dairy cows, beef cattle, sporting animals), which have significant scientific value (e.g., captive or free specimens of endangered species), or which otherwise have value. In addition, as a general matter, the subjects for treatment with the methods of the present invention need not present indications for treatment with the agents of the invention other than those indications associated with need for modulation of angiogenesis. That is, the subjects for treatment are expected to be otherwise free of indications for treatment with the hedgehog therapeutic agents of the invention.

One of ordinary skill in the medical or veterinary arts is trained to recognize subjects which may need modulation of angiogenesis . In particular, clinical and non-clinical trials, as well as accumulated experience, relating to the presently disclosed and other methods of treatment, are expected to inform the skilled practitioner in deciding whether a given subject is in need of modulation and whether any particular treatment is best suited to the subject's needs, including treatment according to the present invention.

VIII. Utilities, Formulations and Methods of Treatment

A. General

We show that hedgehog receptor (ptcl) is normally expressed in the vasculature. We used a mouse which carries the lacZ reporter gene under the control of the endogenous ptc 1 promotor to determine the expression of ptc1 in normal adult animals (Example 1). We further determined that mice injected with hedgehog protein for 3 days

showed no obvious physical or behavioral differences compared to vehicle-treated or untreated littermates. The vascular and cardiovascular staining pattern for ptc1 seen in normal animals intensifies significantly in animals injected with increasing doses of hedgehog protein. Our data show that systemic administration of hedgehog can induce ptc1 upregulation and indicate that these vascular tissues are responsive to hedgehog protein.

We further determined that hedgehog induces neovascularization in a corneal model of angiogenesis (Example 3) as well as a matrigel plug model of angiogenesis (Example 2) . We further found that there was a striking qualitative difference in the appearance of vessels induced by hedgehog compared to VEGF. VEGF induced a fine mesh of capillaries which are short tortous sprouts from the extended branches of the preexisting limbus vessels at the base of the eye. In contrast, hedgehog induced much larger vessels which extended all the way to the pellet and contained numerous anastamoses between the venous and arterial circulation

Moreover, we employed surgical ligation of the femoral artery and removal of a segment of the artery distal to the ligation in mice to induce limb ischemia (Example 5). We found that hedgehog improves recovery from such ischemic limb injury.

In yet another clinically relevant animal model, we placed an ameroid constrictor around the left circumflex coronary artery of pigs. We determined that hedgehog protein or gene therapy can also improve these measures of cardiac perfusion, viability and function following ischemia in this model (Example 6). We determined that hedgehog protein is overexpressed in several human gastrointestinal tumor cell lines compared to normal human gastrointestinal epithelial cells or fibroblasts (Example 7) and that inhibition of hedgehog using, for example, anti-hedgehog blocking antibody, may decrease tumor growth rate and/or tumor angiogenesis (Example 7).

Accordingly, the methods of this invention may employ hedgehog therapeutics or biologically active portions thereof, to promote angiogenesis, such as, to repair damage of myocardial tissue as a result of myocardial infarction. Such methods may also include the repair of the cardiac vascular system after ischemia including the growth of collateral vasculature. Methods utilizing hedgehog therapeutics may be

employed to stimulate the growth of transplanted tissue and collateral vasculature where coronary bypass surgery is performed. Methods may also treat damaged vascular tissue as a result of coronary artery disease and peripheral or central nervous system vascular disease or ischemia.

Methods of the invention may also promote wound healing, particularly to re-vascularize damaged tissues or stimulate collateral blood flow during ischemia and where new capillary angiogenesis is desired. Other methods of the invention may be employed to treat full-thickness wounds such as dermal ulcers, including pressure sores, venous ulcers, and diabetic ulcers. In addition, methods employing hedgehog therapeutics may be employed to treat full-thickness burns and injuries where a skin graft or flap is used to repair such burns and injuries. Such hedgehog therapeutics may also be employed for use in plastic surgery, for example, for the repair of lacerations, burns, or other trauma. In urology, methods of the invention may assist in recovery of erectile function. In the field of female reproductive health, methods of the invention may assist in the modulation of menstruation, ovulation, endometrial lining formation and maintanence, and placentation.

Since angiogenesis is important in keeping wounds clean and non-infected, methods may be employed in association with surgery and following the repair of cuts. They may also be employed for the treatment of abdominal wounds where there is a high risk of infection. Methods using hedgehog therapeutics described herein may be employed for the promotion of endothelialization in vascular graft surgery. In the case of vascular grafts using either transplanted or synthetic material, hedgehog therapeutics can be applied to the surface of the graft or at the junction to promote the growth of vascular smooth muscle and adventitial cells in conjunction with endothelial cells.

Methods of the invention may also be employed to coat artificial prostheses or natural organs which are to be transplanted in the body to minimize rejection of the transplanted material and to stimulate vascularization of the transplanted materials and may also be employed for vascular tissue repair, for example, that occurring during arteriosclerosis and required following balloon angioplasty where vascular tissues are

damaged. Specifically, methods of the invention may be employed to promote recovery from arterial wall injury and thereby inhibit restenosis.

Nucleic acid sequences encoding hedgehog therapeutics may also be employed for in vitro purposes related to scientific research, synthesis of DNA and manufacture of DNA vectors, and for the production of diagnostics and therapeutics to treat human disease. For example. methods of the invention may involve in vitro culturing of vascular smooth muscle cells, fibroblasts, hematopoietic cells, muscle, myotendonous junction, bone or cartilage- derived cells and other mesenchymal cells, where a hedgehog therapeutic is added to the conditional medium in a concentration from 10 ng/ml to 20 ug/ml.

Antagonistic hedgehog therapeutics may be employed to limit angiogenesis necessary for solid tumor metastasis. The identification of antagonists can be used for the generation of certain inhibitors of vascular endothelial growth factor. Since angiogenesis and neovascularization are essential steps in solid tumor growth, inhibition of angiogenic activity of the vascular endothelial growth factor is very useful to prevent the further growth, retard, or even regress solid tumors. Gastrointestinal tumors and gliomas are also a type of neoplasia which may be treated with the antagonists of the present invention.

In addition to these disorders, the antagonists may also be employed to treat retinopathy associated with diabetes, rheumatoid arthritis, osteoarthritis, macular degeneration, glaucoma, Keloid formation, ulcerative colitis, Krohn's disease, psoriasis, and other conditions caused are exacerbated by increased angiogenic activity. The antagonists may be employed in a composition with a pharmaceutically acceptable carrier, e.g., as described herein.

These therapeutic agents may be administered by any route which is compatible with the particular agent employed. The hedgehog therapeutic agents of the invention may be provided to an individual by any suitable means, preferably directly (e.g., locally, as by injection or topical administration to a tissue locus) or systemically (e.g., parenterally or orally). Where the agent is to be provided parenterally, such as by intravenous, intraarterial, subcutaneous, or intramuscular, administration, the agent

preferably comprises part of an aqueous solution. The solution is physiologically acceptable so that in addition to delivery of the desired agent to the subject, the solution does not otherwise adversely affect the subject's electrolyte and/or volume balance. The aqueous medium for the hedgehog therapeutic may comprise normal physiologic saline (e.g., 9.85% NaCl, 0.15M, pH 7-7.4).

The hedgehog therapeutics are preferably administered as a sterile pharmaceutical composition containing a pharmaceutically acceptable carrier, which may be any of the numerous well known carriers, such as water, saline, phosphate buffered saline, dextrose, glycerol, ethanol, and the like, or combinations thereof. The compounds of the present invention may be used in the form of pharmaceutically acceptable salts derived from inorganic or organic acids and bases. Included among such acid salts are the following: acetate, adipate, alginate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, citrate, camphorate, camphorsulfonate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, glucoheptanoate, glycerophosphate, hemisulfate, heptanoate, hexanoate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxyethanesulfonate, lactate, maleate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, oxalate, pamoate, pectinate, persulfate, 3-phenyl-propionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, tosylate and undecanoate. Base salts include ammonium salts, alkali metal salts, such as sodium and potassium salts, alkaline earth metal salts, such as calcium and magnesium salts, salts with organic bases, such as dicyclohexylamine salts, N-methyl-D-glucamine, tris(hydroxymethyl)methylamine and salts with amino acids such as arginine, lysine, and so forth. Also, the basic nitrogen-containing groups can be quaternized with such agents as lower alkyl halides, such as methyl, ethyl, propyl, and butyl chloride, bromides and iodides; dialkyl sulfates, such as dimethyl, diethyl, dibutyl and diamyl sulfates, long chain halides such as decyl, lauryl, myristyl and stearyl chlorides, bromides and iodides, aralkyl halides, such as benzyl and phenethyl bromides and others. Water or oil-soluble or dispersible products are thereby obtained.

Pharmaceutical compositions of hedgehog therapeutics comprise any of the compounds of the present invention, or pharmaceutically acceptable derivatives thereof,

together with any pharmaceutically acceptable carrier. The term "carrier" as used herein includes acceptable adjuvants and vehicles. Pharmaceutically acceptable carriers that may be used in the pharmaceutical compositions of this invention include, but are not limited to, ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts or electrolytes, such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, polyethylene glycol, sodium carboxymethylcellulose, polyacrylates, waxes, polyethylene-polyoxypropylene-block polymers, polyethylene glycol and wool fat.

According to this invention, the pharmaceutical compositions may be in the form of a sterile injectable preparation, for example a sterile injectable aqueous or oleaginous suspension. This suspension may be formulated according to techniques known in the art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed including synthetic mono- or di-glycerides. Fatty acids, such as oleic acid and its glyceride derivatives are useful in the preparation of injectables, as do natural pharmaceutically-acceptable oils, such as olive oil or castor oil, especially in their polyoxyethylated versions. These oil solutions or suspensions may also contain a long-chain alcohol diluent or dispersant.

Controlled release administration of a particular hedgehog therapeutic may be useful. For example, the therapeutic may be administered using intravenous infusion, an implantable osmotic pump, a transdermal patch, liposomes, or other modes of administration. In one embodiment, a pump may be used [Langer et al., eds., Medical Applications of Controlled Release, CRC Pres., Boca Raton, Fla. (1974); Sefton, CRC

Crit. Ref. Biomed. Eng., 14:201 (1987); Buchwald et al., Surgery, 88:507 (1980); Saudek et al., N. Engl. J. Med., 321:574 (1989)]. In another embodiment, polymeric materials can be used [see, Langer, 1974, supra; Sefton, 1987, supra; Smolen et al., eds., Controlled Drug Bioavailability, Drug Product Design and Performance, Wiley, N.Y. (1984); Ranger et al., J. Macromol. Sci. Rev. Macromol. Chem., 23:61 (1983); see also Levy et al., Science, 228:190 (1985); During et al., Ann. Neurol., 25:351 (1989); Howard et al., J. Neurosurg., 71:105 (1989)]. In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, e.g., a tumor, thus requiring only a fraction of the systemic dose [see. e.g., Goodson, in Medical Applications of Controlled Release, vol. 2, pp. 115-138 (1984)]. Other controlled release systems are discussed in the review by Langer, Science, 249:1527-1533 (1990). In another embodiment, the therapeutic compound can be delivered in a vesicle, in particular a liposome (see Langer, 1990, supra); Treat et al., in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, pp. 317-327; see generally id.).

B. Oral Delivery

Contemplated for use herein are oral solid dosage forms, which are described generally in Martin, Chapter 89, 1990, supra, which is herein incorporated by reference. Solid dosage forms include tablets, capsules, pills, troches or lozenges, cachets or pellets. Also, liposomal or proteinoid encapsulation may be used to formulate the present compositions (as, for example, proteinoid microspheres reported in U.S. Pat. No. 4,925,673). Liposomal encapsulation may be used and the liposomes may be derivatized with various polymers (e.g., U.S. Pat. No. 5,013,556). A description of possible solid dosage forms for the therapeutic is given by Marshall, in Modern Pharmaceutics, Chapter 10, Banker and Rhodes ed., (1979), herein incorporated by reference. In general, the formulation will include the therapeutic (or chemically modified form), and inert ingredients which allow for protection against the stomach environment, and release of the biologically active material in the intestine.

For the protein (or derivative) the location of release may be the stomach, the small intestine (the duodenum, the jejunem, or the ileum), or the large intestine. One skilled in the art has available formulations which will not dissolve in the stomach, yet will release the material in the duodenum or elsewhere in the intestine. Preferably, the release will avoid the deleterious effects of the stomach environment, either by protection of the protein (or derivative) or by release of the biologically active material beyond the stomach environment, such as in the intestine. To ensure full gastric resistance, a coating impermeable to at least pH. 5.0 is essential. Examples of the more common inert ingredients that are used as enteric coatings are cellulose acetate trimellitate (CAT), hydroxypropylmethylcellulose phthalate (HPMCP), HPMCP 50, HPMCP 55, polyvinyl acetate phthalate (PVAP), Eudragit L30D, Aquateric, cellulose acetate phthalate (CAP), Eudragit L, Eudragit S, and Shellac. These coatings may be used as mixed films. A coating or mixture of coatings can also be used on tablets, which are not intended for protection against the stomach. This can include sugar coatings, or coatings which make the tablet easier to swallow. Capsules may consist of a hard shell (such as gelatin) for delivery of dry therapeutic i.e. powder; for liquid forms, a soft gelatin shell may be used. The shell material of cachets could be thick starch or other edible paper. For pills, lozenges, molded tablets or tablet triturates, moist massing techniques can be used.

The therapeutic can be included in the formulation as fine multiparticulates in the form of granules or pellets of particle size about 1 mm. The formulation of the material for capsule administration could also be as a powder, lightly compressed plugs or even as tablets. The therapeutic could be prepared by compression. Colorants and flavoring agents may all be included. For example, the protein (or derivative) may be formulated (such as by liposome or microsphere encapsulation) and then further contained within an edible product, such as a refrigerated beverage containing colorants and flavoring agents. One may dilute or increase the volume of the therapeutic with an inert material. These diluents could include carbohydrates, especially mannitol, alpha - lactose, anhydrous lactose, cellulose, sucrose, modified dextrans and starch. Certain inorganic salts may be also be used as fillers including calcium triphosphate, magnesium

carbonate and sodium chloride. Some commercially available diluents are Fast-Flo, Emdex, STA-Rx 1500, Emcompress and Avicell. Disintegrants may be included in the formulation of the therapeutic into a solid dosage form. Materials used as disintegrants include but are not limited to starch including the commercial disintegrant based on starch, Explotab. Sodium starch glycolate, Amberlite, sodium carboxymethylcellulose, ultramylopectin, sodium alginate, gelatin, orange peel, acid carboxymethyl cellulose, natural sponge and bentonite may all be used. Another form of the disintegrants are the insoluble cationic exchange resins. Powdered gums may be used as disintegrants and as binders and these can include powdered gums such as agar, Karaya or tragacanth. Alginic acid and its sodium salt are also useful as disintegrants. Binders may be used to hold the therapeutic agent together to form a hard tablet and include materials from natural products such as acacia, tragacanth, starch and gelatin. Others include methyl cellulose (MC), ethyl cellulose (EC) and carboxymethyl cellulose (CMC). Polyvinyl pyrrolidone (PVP) and hydroxypropylmethyl cellulose (HPMC) could both be used in alcoholic solutions to granulate the therapeutic. An antifrictional agent may be included in the formulation of the therapeutic to prevent sticking during the formulation process. Lubricants may be used as a layer between the therapeutic and the die wall, and these can include but are not limited to: stearic acid including its magnesium and calcium salts, polytetrafluoroethylene (PTFE), liquid paraffin, vegetable oils and waxes. Soluble lubricants may also be used such as sodium lauryl sulfate, magnesium lauryl sulfate, polyethylene glycol of various molecular weights, and Carbowax 4000 and 6000. Glidants that might improve the flow properties of the drug during formulation and to aid rearrangement during compression might be added. The glidants may include starch, talc, pyrogenic silica and hydrated silicoaluminate.

To aid dissolution of the therapeutic into the aqueous environment, a surfactant might be added as a wetting agent. Surfactants may include anionic detergents such as sodium lauryl sulfate, dioctyl sodium sulfosuccinate and dioctyl sodium sulfonate. Cationic detergents might be used and could include benzalkonium chloride or benzethomium chloride. The list of potential nonionic detergents that could be included in the formulation as surfactants are lauromacrogol 400, polyoxyl 40 stearate,

polyoxyethylene hydrogenated castor oil 10, 50 and 60, glycerol monostearate, polysorbate 40, 60, 65 and 80, sucrose fatty acid ester, methyl cellulose and carboxymethyl cellulose. These surfactants could be present in the formulation of the protein or derivative either alone or as a mixture in different ratios. Additives which potentially enhance uptake of the protein (or derivative) are for instance the fatty acids oleic acid, linoleic acid and linolenic acid.

C. Pulmonary Delivery

Also contemplated herein is pulmonary delivery of the present proteins (or derivatives thereof). The protein (or derivative) is delivered to the lungs of a mammal while inhaling and traverses across the lung epithelial lining to the blood-stream. Other reports of this include Adjei et al., Pharmaceutical Research, 7(6):565-569 (1990); Adjei et al., International Journal of Pharmaceutics, 63:135-144 (1990) (leuprolide acetate); Braquet et al., Journal of Cardiovascular Pharmacology, 13(suppl. 5):143-146 (1989) (endothelia-1); Hubbard et al., Annals of Internal Medicine, 3(3):206-212 (1989) (alpha 1-antitrypsin); Smith et al., J. Clin. Invest., 84:1145-1146 (1989) (alpha 1-proteinase); Os wein et al., "Aerosolization of Proteins", Proceedings of Symposium on Respiratory Drug Delivery II, Keystone, Colo., (March 1990) (recombinant human growth hormone); Debs et al., J. Immunol., 140:3482-3488 (1988) (interferon- gamma and tumor necrosis factor alpha) and Platz et al., U.S. Pat. No. 5,284,656 (granulocyte colony stimulating factor). Contemplated for use in the practice of this invention are a wide range of mechanical devices designed for pulmonary delivery of therapeutic products, including but not limited to nebulizers, metered-dose inhalers, and powder inhalers, all of which are familiar to those skilled in the art.

Some specific examples of commercially available devices suitable for the practice of this invention are the Ultravent nebulizer, manufactured by Mallinckrodt, Inc., St. Louis, Mo.; the Acorn II nebulizer, manufactured by Marquest Medical Products, Englewood, Colo.; the Ventolin metered-dose inhaler, manufactured by Glaxo Inc., Research Triangle Park, N.C.; and the Spinhaler powder inhaler, manufactured by Fisons Corp., Bedford, Mass. All such devices require the use of formulations suitable

for the dispensing of protein (or derivative). Typically, each formulation is specific to the type of device employed and may involve the use of an appropriate propellant material, in addition to the usual diluents, adjuvants and/or carriers useful in therapy. Also, the use of liposomes, microcapsules or microspheres, inclusion complexes, or other types of carriers is contemplated. Chemically modified protein may also be prepared in different formulations depending on the type of chemical modification or the type of device employed.

Formulations suitable for use with a nebulizer, either jet or ultrasonic, will typically comprise protein (or derivative) dissolved in water at a concentration of about 0.1 to 25 mg of biologically active protein per ml of solution. The formulation may also include a buffer and a simple sugar (e.g., for protein stabilization and regulation of osmotic pressure). The nebulizer formulation may also contain a surfactant, to reduce or prevent surface induced aggregation of the protein caused by atomization of the solution in forming the aerosol.

Formulations for use with a metered-dose inhaler device will generally comprise a finely divided powder containing the protein (or derivative) suspended in a propellant with the aid of a surfactant. The propellant may be any conventional material employed for this purpose, such as a chlorofluorocarbon, a hydrochlorofluorocarbon, a hydrofluorocarbon, or a hydrocarbon, including trichlorofluoromethane, dichlorodifluoromethane, dichlorotetrafluoroethanol, and 1,1,1,2-tetrafluoroethane, or combinations thereof. Suitable surfactants include sorbitan trioleate and soya lecithin. Oleic acid may also be useful as a surfactant.

Formulations for dispensing from a powder inhaler device will comprise a finely divided dry powder containing protein (or derivative) and may also include a bulking agent, such as lactose, sorbitol, sucrose, or mannitol in amounts which facilitate dispersal of the powder from the device, e.g., 50 to 90% by weight of the formulation. The protein (or derivative) should most advantageously be prepared in particulate form with an average particle size of less than 10 mum (or microns), most preferably 0.5 to 5 mum, for most effective delivery to the distal lung.

D. Dosages

For all of the above molecules, as further studies are conducted, information will emerge regarding appropriate dosage levels for treatment of various conditions in various patients, and the ordinary skilled worker, considering the therapeutic context, age and general health of the recipient, will be able to ascertain the proper dosage. Generally, for injection or infusion, dosage will be between 0.01 mu. g of biologically active protein/kg body weight, (calculating the mass of the protein alone, without chemical modification), and 10 mg/kg (based on the same). The dosing schedule may vary, depending on the circulation half-life of the protein or derivative used, whether the polypeptide is delivered by bolus dose or continuous infusion, and the formulation used.

E. Administration with Other Compounds

For therapy associated with modulating angiogenesis, one may administer the present hedgehog therapeutics (or derivatives) in conjunction with one or more pharmaceutical compositions used for treating other clinical complications of the need for angiogenic modulation, such as those used for treatment of cancer (e.g., chemotherapeutics), cachexia, high blood pressure, high cholesterol, and other adverse conditions. Administration may be simultaneous or may be in seriatim. Similarly, one may administer more than one hedgehog therapeutic (or derivatives), having the same or differing mode of action, to attain an additive or synergistic effect on angiogenesis.

F. Nucleic Acid-Based Therapeutic Treatment

Nucleic acid sequences encoding an antagonisitic hedgehog therapeutic could be introduced into human tumor or blood vessel cells to develop gene therapy. Similarly, nucleic acid sequences encoding an agonistic hedgehog therapeutic could be introduced into human cells as a gene therapy based treatment.

In one embodiment, a nucleic acid sequence encoding a hedgehog therapeutic is introduced in vivo in a viral vector. Such vectors include an attenuated or defective DNA virus, such as but not limited to herpes simplex virus (HSV), papillomavirus, Epstein Barr virus (EBV), adenovirus, adeno-associated virus (AAV), and the like.

Defective viruses, which entirely or almost entirely lack viral geries, are preferred. Defective virus is not infective after introduction into a cell. Use of defective viral vectors allows for administration to cells in a specific, localized area, without concern that the vector can infect other cells. Thus, adipose tissue can be specifically targeted. Examples of particular vectors include, but are not limited to, a defective herpes virus 1 (HS V 1) vector [Kaplitt et al., Molec. Cell. Neurosci., 2:320-330 (1991)], an attenuated adenovirus vector, such as the vector described by Stratford-Perricaudet et al., J. Clin. Invest., 90:626-630 (1992), and a defective adeno-associted virus vector [Samulski et al., J. Virol., 61:3096-3101 (1987); Samulski et al., J. Virol., 63:3822-3828 (1989)]. In another embodiment, the nucleic acid can be introduced in a retroviral vector, e.g., as described in Anderson et al., U.S. Pat. No. 5,399,346; Mann et al., Cell, 33:153 (1983); Temin et al., U.S. Pat. No. 4,650,764; Temin et al., U.S. Pat. No. 4,980,289; Markowitz et al., J. Virol., 62:1120 (1988); Temin et al., U.S. Pat. No. 5,124,263; International Patent Publication No. WO 95/07358, published Mar. 16, 1995, by Dougherty et al.; and Kuo et al., Blood, 82:845 (1993).

Alternatively, the vector can be introduced in vivo by lipofection. For the past decade, there has been increasing use of liposomes for encapsulation and transfection of nucleic acids in vitro. Synthetic cationic lipids designed to limit the difficulties and dangers encountered with liposome mediated transfection can be used to prepare liposomes for in vivo transfection of a gene encoding a marker [Felgner et al., Proc. Natl. Acad. Sci. USA, 84:7413-7417 (1987); see Mackey et al., Proc. Natl. Acad. Sci. USA, 85:8027-8031 (1988)]. The use of cationic lipids may promote encapsulation of negatively charged nucleic acids, and also promote fusion with negatively charged cell membranes [Felgner et al., Science, 337:387-388 (1989)]. The use of lipofection to introduce exogenous genes into specific organs in vivo has certain practical advantages. Molecular targeting of liposomes to specific cells represents one area of benefit. It is clear that directing transfection to particular cell types would be particularly advantageous in a tissue with cellular heterogeneity, such as the pancreas, liver, kidney, and brain. Lipids may be chemically coupled to other molecules for the purpose of targeting (see Mackey et al., 1988, supra). Targeted peptides, e.g., hormones or

neurotransmitters, and proteins such as antibodies, or non-peptide molecules could be coupled to liposomes chemically.

It is also possible to introduce the vector in vivo as a naked DNA plasmid. Naked DNA vectors for gene therapy can be introduced into the desired host cells by methods known in the art, e.g., transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, use of a gene gun, or use of a DNA vector transporter (see, e.g., Wu et al., J. Biol. Chem., 267:963-967 (1992); Wu et al., J. Biol. Chem., 263:14621-14624 (1988); Hartmut et al., Canadian Patent Application No. 2,012,311, filed Mar. 15, 1990).

It is also possible to introduce the vector in vivo in conjuction with a catheter or other device. See Vale et al., 1999: Kornowski et al., 2000.

H. Diagnostics

A diagnostic method useful in the present invention comprises examining a cellular sample or medium by means of an assay including an effective amount of an antagonist to a hedgehog protein, such as an anti-hedgehog antibody homolog, preferably an affinity-purified polyclonal antibody, and more preferably a mAb. In addition, it is preferable for the anti-hedgehog antibody molecules used herein be in the form of Fab, Fab', F(ab)2 or F(v) portions or whole antibody molecules. As previously discussed, patients capable of benefiting from this method include those suffering from cancer or other conditions where abnormal angiogenesis is a characteristic or factor. Methods for isolating hedgehog protein and inducing anti-hedgehog antibodies and for determining and optimizing the ability of anti-hedgehog antibodies to assist in the examination of the target cells are all well-known in the art.

The present invention will be illustrated by the following, non-limiting examples. These are described in further detail in the pending publication, Pola et al., 2001, Nature Medicine, incorporated herein.

Example 1. Hedgehog Responsive Cells in Normal Vasculature

The Expression of Hedgehog Receptor in Normal Vasculature

The hedgehog receptor which is coupled directly to the hedgehog signalling pathway is patched 1 (ptc1). In addition to being the primary hedgehog receptor in the signalling pathway, ptc1 gene expression is also induced by signalling through the hedgehog pathway. The expression of the ptc1 gene in cells can thus indicate that the cell is potentially responsive to hedgehog proteins and can also show that the cell is in the process of responding to hedgehog stimulation. We used a mouse which carries the lacZ reporter gene under the control of the endogenous ptc1 promotor to determine the expression of ptc1 in normal adult animals

Ptcl-lacZ mice carry a non disruptive insertion of the lacZ reporter gene containing a nuclear localization signal upstream of the ptc1 coding region. LacZ expression corresponds to ptc1 expression (Goodrich et al., 1997; M. Scott, Ontogeny, personal communication). Ptc1 expression does not appear to be altered by LacZ insertion and expression corresponds to ptc1 expression in embryos (M. Scott, Ontogeny, personal communication). Heterozygous Ptcl-lacZ mice and their wild type littermate controls are generated by mating heterozygote lacZ positive males with standard C57BL/6J female mice (Taconic, Germantown, NY). Adult Ptcl-lacZ mice were fixed by cardiac perfusion followed by drop fixation of heart or vascular tissues for 1-2 hours in 0.2% gluteraldehyde, 5mM EDTA, 2mM $MgCl_2$, O.1M sodium phosphate, pH8. Pup tissues and small tissues were directly drop fixed in gluteraldehyde for 1-2 hours. Following fixation, the tissues were washed 3 times for 20-30 min in 2mM $MgCl_2$, 0.01 deoxycholate, 0.02% NP40, 50mM sodium phosphate pH8. The tissues were then stained overnight at 37°C in lmg/ml 5-Bromo-4-chloro-3-indolyl -D-galactopyranoside (Xgal) (Sigma, St. Louis, MO), 5 mM potassium ferricyanide, 5mM potassium ferrocyanide, 2mM $MgCl_2$, 0.01 % deoxycholate, 0.02% NP40, 50mM sodium phosphate pH8. The tissues were visualized either as whole mounts or embedded in paraffin and prepared as light eosin-stained 5 micron sections.

Patched 1 is expressed in the endothelial cells of the aorta, some vascular smooth muscle cells (vSMC) and adventitial fibroblasts of the aorta (photomicrographs not presented here). In addition, coronary vasculature and cardiomyocytes of the atria and

ventricles also express ptcl. These expression patterns suggest that cells in normal vascular and cardiovascular tissues may be responsive to or responding to hedgehog.

Normal Vasculature and Cardiovascular Tissues are Hedgehog Responsive

We determined that normal vascular and cardiovascular tissues are indeed responsive to exogenous hedgehog administration by injecting Ptcl-lacZ mice systemically with hedgehog. Ptcl-lacZ mice were injected daily subcutaneously with the indicated amounts of polyethylene glycol 20,000-conjugated A192C sonic hedgehog n-terminal protein (PEG-Shh) (Pepinsky et al, 2000) or its vehicle (PBS). This form of the protein also contains a mutation of the n-terminal cysteine residue to isoleucine-isoleucine which significantly improves the specific activity of hedgehog protein (Pepinsky et al, 1998; Taylor et al, in prep).

Mice injected with hedgehog protein for 3 days showed no obvious physical or behavioural differences compared to vehicle-treated or untreated littermates. Specifically, Ptcl-lacZ mice were injected (s.c.) once daily with PEG-Shh for 3 days starting at postnatal day 6 then sacrificed at postnatal day 9; selected organs were dissected and whole mount stained by X-Gal histochemistry. Mice were treated with vehicle, 3mg/kg PEG-Shh or 6mg/kg PEG-Shh for 3 days and were sacrificed on the fourth day. Vascular and cardiovascular tissues were dissected and whole-mount stained with Xgal. The vascular and cardiovascular staining pattern for ptcl seen in normal animals intensifies significantly in animals injected with increasing doses of hedgehog protein (data not presented here). Whole mount Xgal staining of the coronary arteries, atria and ventricles are increased in a dose dependent manner in the hearts and in the aortic wall of the Ptcl-lacZ mice injected with hedgehog. In contrast, wild type littermate mice injected with the highest dose of hedgehog (6mg/kg) show no staining suggesting that the staining seen in the Ptcl-lacZ animals is not due to endogenous betagalactosidase. Histological sections of these tissues show that the lacZ positive cells in the Ptcl-lacZ mice treated with hedgehog are similar to those which are positive in the vehicle-injected group and in normal adult hearts and aortas from untreated animals. Though the same type of cells appear to stain with Xgal in the treated animals, there

appears to be an increase in the number of these cells especially in the adventitia. These data show that systemic administration of hedgehog can induce ptc1 upregulation and indicate that these vascular tissues are responsive to hedgehog protein.

Example 2: Hedgehog Induces Neovascularization in Matrigel Plug Model of Angiogenesis

Hedgehog was also found to induce angiogenesis in the subcutaneous matrigel plug assay (Passaniti et al., 1992). Doses of 2 to 10ug/ml of octyl, myr, PEG II or II-Fc fusion forms of human recombinant Shh were prepared in 0.5ml of matrigel containing 40 IU/ml of heparin and injected subcutaneously into C57BL6 mice (3-5mo. old, 5 mice/treatment group). The mice were sacrificed between 6-7 days after injection and the matrigel plug was dissected for visual inspection and histological analysis. Plugs containing hedgehog induced significant angiogenesis in the plug and surrounding tissue in 4 of 6 plugs at 2ug/ml and 5 of 6 plugs at 10ug/ml whereas only 2 of 9 vehicle containing plugs showed any evidence of angiogenesis (data not presented here). Recombinant human bFGF, a known angiogenic protein, also showed significant hemoglobin content in 3 of 5 implants (data not shown). The results of the matrigel plug support the finding that hedgehog can induce angiogenesis in vivo.

Example 3: Hedgehog Induces Neovascularization in Corneal Model of Angiogenesis

The mouse cornea is avascular and can be used to demonstrate angiogenic activity by measuring the amount of vessel growth into this avascular tissue after surgical placement of a polymer pellet containing an angiogenic substance or growth factor into the cornea (Kenyon et al., 1996; Asahara et al., 1997). To confirm the angiogenic activity of hedgehog in another well accepted model of angiogenesis, we tested the ability of hedgehog protein to induce neovascularization in the mouse corneal model pocket model of angiogenesis.

Animals were anesthetized by pentobarbital intraperitoneal injection (160 mg/kg). Corneal pockets were created in the eyes of each mouse and a 0.34 X 0.34 mm

sucrose albumin sulfate (Bukh Meditec, Vaerlose, DK) pellet coated with hydron polymer type NCC (Interferon Sciences, New Brunswick, NJ) containing 1 of the agents indicated below was implanted into the corneal pocket. C57BL/fJ mice were divided into 5 groups: control buffer alone; VEGF 300 ng/pellet; Myr-Shh vehicle alone; Myr-Shh 1.5 microg/pellet 39; Myr-Shh+VEGF (1.5 microg/pellet +300 ng/pellet, respectively). Pellets were positioned 1.0 mm from the corneal limbus, and erythromycin ophthalmic ointment (E. Fourera) was applied to each operated eye. The corneas of all mice were routinely examined by slit-lamp biomicroscopy on postoperative day 6 after pellet implantation.

On the same day vessel length and corneal circumferential neovascularity (in degrees) were measured. After completing these measurements, C57BL/6J mice received an intravenous injection of 500 pg of BS-1 lectin FITC-conjugated (Vector Laboratories, Burlingame, CA). Thirty minutes later, the animals were sacrificed. The eyes were enucleated and fixed in 1 % paraformaldehyde solution. After fixation, the corneas were placed on glass slides and examined by fluorescence microscopy. Several C57BL/6J mice in each group did not receive BS-1 lectin injection; instead, the eyes were excised and fixed in 100% methanol solution for immunohistochemical staining.

There was significant neovascular growth in the Shh and in the VEGF groups but not the vehicle-containing pellet groups. There was a striking qualitative difference in the appearance of vessels induced by hedgehog compared to VEGF (photomicrographs not presented here). VEGF induced a fine mesh of capillaries which are short tortous sprouts from the extended branches of the preexisting limbus vessels at the base of the eye. In contrast, hedgehog induced much larger vessels which extended all the way to the pellet and contained numerous anastamoses between the venous and arterial circulation. Histological analysis confirmed that hedgehog induced larger diameter vessels than VEGF. Hedgehog induced vessels often were filled with red blood cells whereas VEGF induced vessels had few or no red blood cells.

Measurements (mean±standard error of the mean) of the VEGF and hedgehog vessels confirmed that hedgehog-induced vessel diameters (mean 33±17um) were significantly larger than VEGF vessel diameters (mean 8±3um) ($p<0.0001$)). The

maximum vessel lengths induced by hedgehog (1020+200um) were also significantly greater than the maximum length of vessels induced by VEGF (700±70um) ($p<0.0001$). The density of vessels induced by hedgehog was slightly lower than the density of vessels in the corneal tissue exposed to VEGF as may be expected from the large number of small capillaries formed by VEGF ($p<0.0001$). All group differences were analysed by ANOVA and differences with $p<0.05$ were considered statistically significant.

In summary, neovascularization induced by Shh was characterized by a statistically significant increase in vessel length, circumferential neovascularity and diameter of the lumens; the mean number of vascular lumens per cross section was higher in the VEGF-treated corneas. Neovascularization induced by Shh+VEGF showed a large variability in the lumen diameter of these vessels ranging from small capillaries (6-7 gm) to large diameter vessels (80 gm). The combination of VEGF and Shh appears to create a composite of characteristics of both VEGF and Shh neovascular growth. These results confirm hedgehog protein can induce angiogenesis in vivo and suggest that hedgehog either alone or in combination with VEGF or other angiogenic growth factors such as bFGF, the angiopoietins and TWEAK [Lynch CN, Wang YC, Lund JK, Chen YW, Leal JA, Wiley SR. TWEAK induces angiogenesis and proliferation of endothelial cells. J Biol Chem. 1999 Mar 26;274(13):8455-9] can have therapeutic utility by inducing functional neovasculature.

Example 4: Biological Activities Induced By Hedgehog -Responsive Mesenchymal Cells

Hedgehog induces stromal fibroblasts and VEGF upregulation in the corneal model of angiogenesis

To determine the mechanism by which Shh induces angiogenesis both Shh and VEGF-stimulated corneas (see Example 3) were excised and X-gal stained as described in Example 1 after fixation of the whole eye for 1 hour in 1 % paraformaldehyde followed by enucleation and fixation of the corneal hemisphere in 1 % paraformaldehyde for 30 minutes. VEGF-induced corneas did not stain with X-gal,

indicating that VEGF does not induce Ptc 1 expression during neovascularization. In contrast, strong X-gal staining was detected in the neovascular regions of Ptcl-lacZ corneas treated with Shh (data not presented here). Histologic analysis following paraffin embedding of X-gal-stained corneas and preparation of immunostained 5um sections with showed that the X-gal positive cells were not endothelial cells or smooth muscle cells, but fibroblasts surrounding the neovessels. Endothelial cell immunostaining was done with a rat monoclonal antibody against mouse CD-31 (Pharmigen, San Diego, CA) followed by a biotinylated goat anti-rat immunoglobulin secondary antibody. Smooth muscle cells and pericytes were identified with a mouse monoclonal antibody against SM a-actin conjugated with alkaline-phosphatase (Sigma, St. Louis, MO) and fibroblasts were identified using an anti-vimentin antibody (Sigma, St. Louis, MO).

We then immunostained the Shh-induced corneas with a rabbit polyclonal anti-VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA) with a biotinylated goat anti-rabbit immunoglobulin as secondary antibody. The results show that VEGF protein is in the fibroblasts and matrix immediately adjacent to the neovascular area. These results suggested that hedgehog may induce resident fibroblasts in the cornea to produce angiogenic factors such as VEGF.

Fibroblasts in vitro respond to hedgehog stimulation by upregulation of Ptcl and angiogenic growth factors

To determine if hedgehog can directly induce fibroblasts to produce VEGF or other angiogenic factors , we treated normal human fibroblasts (CCD37) with Myr-Shh and the ability of fibroblasts to respond was evaluated by competitive RT-PCR for ptc 1 and several angiogenic growth factors. Total RNA was prepared from cells treated as described above using Trizol (Life Technologies, Rockville, MD). Four micrograms of total RNA was used to prepare cDNA using the SuperScriptTM preamplification system (Cat. No. 18089-011, Life Technologies, Rockville, MD). The PCR reaction using buffer reagents from the SuperScriptTM preamplification system (Life Technologies, Rockville, MD) was quantitated with 20S rRNA competitive primers (Ambion). Primers

for the amplification of Ptc 1' were 5'-TCAGGATGCATTTGACAGTGACTGG-3' (SEQ ID NO: 38) and 5'- ACTCCGAGTCGGAGGAATCAGACCC-3' (SEQ ID NO: 39) which are based on ptcl cDNA sequence (GenBank Accession Number U46155). All amplification for Ptcl were done with 25 cycles of 94°C for 30 sec; 55°C for 1 min; 72°C for 1 min. The cDNA from the same cells was also used as a template for VEGF, bFGF, Angiopoietin 1, and Angiopoietin II amplification. The following primer pairs and PCR cycles were used: VEGF: 5'CGAAGTGGTGAAGTTCATGGATG3' (SEQ ID NO: 40) and 5'TTCTGTATCAGTCTTTCCTGGTGAG3' (SEQ ID NO: 41) which are based on the human VEGF cDNA sequence (GenBank Accession Number E15157). VEGF product was amplified with 30 cycles of 94°C for 30 sec; 62°C for 1 min; 72°C for lmin; bFGF: 5'TACAACTTCAAGCAGAAGAG3' (SEQ ID NO: 42) and 5'CAGCTCTTAGCAGACATTGG3' (SEQ ID NO: 43) which is based on the human bFGF cDNA sequence (GenBank Accession Number M27968). bFGF product was amplified with with 25 cycles of 94°C for 30 sec; 62°C for 1 min; 72°C for 1 min; Angiopoietin I 5'CAACACAAACGCTCTGCAGAGAGA3' (SEQ 1D NO: 44) and 5'CTCCAGTTGCTGCTTCTGAAGGAC 3' (SEQ ID NO: 45) which is based on human Angiopoietin I cDNA sequence (GenBank Accession Number U83508). Angiopoietin I product was amplified with 25 cycles of 94°C for 30 sec; 64°C for 90 sec; Angiopoietin II: 5'AGCGACGTGAGGATGGCAGCGTT3' (SEQ ID N0:46) and 5'ATTTCCTGGTTGGCTGATGCTGCTT3' (SEQ ID NO: 47) which are based on human Angiopoietin II cDNA sequence (GenBank Accesion Number AB009865). Angiopoietin II product was amplified with with 32 cycles of 94°C for 30 sec; 64°C for 90 sec. As internal control for sample preparation, gel loading, and random variations in RT-PCR, 18S rRNA primers and 18S rRNA competimers (Ambion, Austin, TX), used to modify 18S cDNA amplification efficiency, were included in each PCR reaction with target gene-specific primers. The linear range of amplification and optimal 18S primer/Competimer ratio was determined for each target gene following the manufacturer's recommendations (Ambion, Austin, TX).

A time course of Shh induction shows that human fibroblast respond to Shh by upregulating the Ptc1 gene (data not shown) indicating that these cells can respond via

the known Hh signalling pathway. Neither human umbilical vein and microvascular endothelial cells respond to Hh (data not shown).

We next found that Hh can upregulate fibroblast expression of angiogenic growth factors, including VEGF, bFGF, Ang-1, and Ang-2 (data not shown). VEGF mRNA from human fibroblasts was significantly increased by Shh: all the three VEGF isoforms (VEGF121, 165, and 189) were strongly upregulated. VEGF 121, 165, and 189 upregulation began at 12 hours and was maximal after 48 hours of incubation of the cells with Shh. No bFGF upregulation was detectable at any time-points. Moreover, quantitative RT-PCR for Ang-1 and Ang-2 showed upregulation of both genes, with maximal increase after 36 hours stimulation. To show that the upregulation of VEGF mRNA correlated with an increase in protein production, the concentration of VEGF165 in cell media was measured by ELISA. Cells were stimulated with recombinant human myristolated Shh protein as described above. At harvest, the cell conditioned media was collected, centrifuged to remove cell debris (15 minutes at 1500xg) and production of VEGF165 protein was evaluated by using an ELISA kit (Quantikine human VEGF, R&D Systems, Minneapolis, MN). Total VEGF protein level underwent a progressive increase following Hh stimulation and a significant upregulation in the VEGF production was detectable at 72 hours (data not shown).

Smooth muscle cells upregulate ptcl and are induced to proliferate in vitro in response to hedgehog

We found that smooth muscle cells can also respond to Hh proteins in vitro. Eighty five percent confluent monolayers of vascular smooth muscle cells (PAC 1) were induced for 2 days with lug/ml of myrShh or an equivalent volume of vehicle in normal media (M 199 complete media with 10% fetal bovine serum). For comparison, primary normal human lung fibroblasts and normal prostate stromal cells were grown in complete FBM and similarly stimulated (Clonetics/Bio-Whittaker, Walkersville, MD). The cells were harvested and RNA from the cells was prepared and analysed by RT-PCR as above. All of these cells showed increased ptc 1 expression following induction with myrShh, but not myrShh vehicle alone suggesting that each of these cell types are

responsive to hedgehog (data not shown). In addition, hedgehog protein induced DNA synthesis in quiescent vascular SMCs and human fibroblasts. PAC-1 (Rothman et al., 1992), WKY (Lemire et al., 1994), primary pulmonary artery SMCs or aortic SMCs (Clonetics/Bio-Whittaker, Walkersville, MD) were plated (5x103/well) in 96 well plates and allowed to adhere for 2-3 hours in 0.18ml of complete media (M 199 with 10% fetal bovine serum for PAC 1 cells, DMEM with 10% fetal bovine serum for WKY cells or smGM-2 for primary human pulmonary artery or aortic SMCs). The cells were then starved for 18- 24 hours in complete media with 0.5% fetal bovine serum. Quiescent cells were stimulated with 0.1 to 40ug/ml of Hh proteins in 0.2ml starvation media for 48 hours after which the cells were pulse labeled with 4.5uCi/ml 3H-thymidine (Amersham,) for 4-8 hours at 37°C. The media was then removed, the cells washed with PBS then trypsinized. 3H-thymidine uptake into cells was determined by scintillation counting using a 1205 Betaplate counter (Wallac, Gaithersburg, MD). Vascular SMCs showed increased 3H-thymidine uptake 3 to 4-fold when induced by either myrShh (myristylated Sonic hedgehog) Dhh or basicFGF (obtained from Upstate Biotechnology, Lake Placid, NY).

These results show that both SMCs and fibroblasts respond to hedgehog. Although no smooth muscle cells were found in the hedgehog-stimulated corneas (see Example 1 and 4), the responsiveness of SMCs to Hh in vitro correlates well to normal ptc1 expression and increased ptc1 in the response by normal vascular SMCs to systemically administered Hh protein (See Example 3).

Example 5: Hedgehog Improves Recovery from Ischemic Limb Injury

Peripheral vascular disease caused by atherosclerosis and/or diabetes can be modeled in rodents and rabbits by surgical ligation of the femoral artery and removal of a segment of the artery distal to the ligation (Takeshita et al., 1994 and 1996; Rivard et al., 1999; Couffinhal et al., 1999). The limb ischemia produced by the ligation also results in limb neuropathy (Schratzberger et al., 2000). Ischemic injury of healthy animals and humans activates a number of pathways which subsequently induce the regeneration and recovery of the damaged tissue. For example, VEGF is induced in

response to hindlimb ischemia and can accelerate recovery when given pharmacologically following this ischemic insult (Schratzberger et al., 2000). We investigated the possibility that the hedgehog pathway is activated in response to limb ischemia in normal animals and is beneficial both in the endogenous and pharmacological settings to revascularization and recovery from ischemic neuropathy.

The expression of ptc1 following hindlimb ischemia was investigated in 3-4 month old Ptcl-lacZ mice (Rivard et al., 1999). The mice were anesthetized with pentobarbital (160mg/kg i.p.) and an incision was made in the skin overlying the middle portion of the left hindlimb. Both the proximal end of the femoral artery and the distal portion of the saphenous artery were ligated and the artery and all side branches were dissected free and excised. The skin was closed with a surgical stapler and the animals were allowed to recover. The mice were either left untreated or injected daily or every other day i.m. in the ischemic limb with lmg/kg of II-Shh/mouse IgGI Fc fusion protein. Seven days after induction of ischemia, the animals were sacrificed and the upper hindlimb was isolated and whole mount stained with Xgal. Comparison of the contralateral upper hindlimbs (right) to the ischemic hindlimbs (left) shows a significant upregulation of ptc1 expression (data not shown). Ischemia alone induced upregulation of ptcl expression in the ischemic limb and increasing frequency of hedgehog injection further increased ptc1 expression in the ischemic limb muscle. Histological sections of the ischemic and control hindlimb muscle showed muscle fiber degeneration and edema in the ischemic versus nonischemic tissue (data not shown). In addition, the ischemic muscle has a number of ptcl-expressing (Xgal-stained) stromal cells in the interstitial areas between the muscle fibers. These cells which appear to be responding to hedgehog were shown to be fibroblasts identified by costaining with vimentin and X-gal or monocytes/macrophages identified by costaining with the moma2 antibody and X-gal (see Example 4 for Methods). These results show that the hedgehog pathway may be part of the normal response to ischemia which may be augmented by pharmacological administration of hedgehog protein.

The relevance of hedgehog upregulation following ischemia is determined by inhibiting hedgehog action with a blocking antibody to hedgehog. Unilateral hindlimb

ischemia was induced in normal mice (C57BL6, 3-4months of age, female). The mice are treated with 10mg/kg daily 3 days prior to ischemia and 2.5-5mg/kg every 3 days following ischemia for 3 weeks with either the blocking antibody to hedgehog (5E1, Developmental Studies Hybridoma Bank, Karen Jensen, Department of Biological Sciences, The University of Iowa, 007 Biology Building East, Iowa City, IA 52242, tel: (319)335-3826, fax: (319)335-2077, 5E1 available for order on website: www.uiowa.edu/-dshbwww/l*ndex.html, e-mail: dshb@uiowa.edu) or an isotype matched control mouse monoclonal antibody.

The vascular perfusion of the ischemic vs contralateral limb is assessed at days 4, 7, 14, 21 and 28 days by lasar doppler (Lisca, Inc. laser Doppler perfusion imager system) (Rivard et al., 1999). Nerve vascular perfusion is determined by exposing the sciatic nerve and scanning the nerve surface area using lasar doppler or by injection of Fluoresceinated-BS 1 lectin (Vector Laboratories, Burlingame, CA) 30 minutes prior to sacrifice and visualizing the vaso nervorum by whole mount fluorescence microscopy postmortem (as described above). Vascular density is assessed at these times by histological staining for CD31 positive vasculature in sections (anti-murine CD31, Pharmingen, San Diego, CA) (Rivard et al., 1999). Neuropathy is assessed at these time points by nerve conduction measurements of the sciatic/peroneal nerves using standard orthodromic surface recording techniques and a Teca TD-10 portable recording system (Oxford Instruments, Concord, MA). Angiogenesis as measured both by vascular perfusion or vascular density is decreased in ischemic limbs of animals treated with hedgehog blocking antibody, 5E1, compared to ones treated with the isotype matched control, 1E6. Nerve conduction measurements are also decreased in 5E1-treated mice compared to control antibody-treated mice. Finally, nerve vascular perfusion is decreased in the 5E1-treated mice. These results suggest that the upregulation of the hedgehog pathway following ischemia is a beneficial compensatory response to ischemic injury.

The utility of treating ischemia by activating the hedgehog pathway is tested in aged mice (>2yrs old) or apoE null mice with surgically induced limb ischemia since these mice are deficient in their repair and regeneration processes following limb

ischemia. These mice are made ischemic then injected (i.v., i.p., s.c. or i.m.) with doses ranging from 0-10mg/kg of hedgehog protein or equivalent volumes of vehicle control or control protein beginning on the day of surgery and with a frequency of daily to 3 times per week. The vascular perfusion, vascular density and neuronal conduction and neuronal vascularity (vaso nervorum) of the ischemic vs contralateral limb are assessed at days 4, 7, 14, 21 and 28 postsurgery as described above. The results show that hedgehog-treated animals show significant improvements in vascular perfusion, vascular density as well as motor nerve conduction and their vaso nervorum compared to control treated animals (data not presented).

Hedgehog can also be delivered using gene therapy. Either full length or soluble Nterminal Shh adenovirus (10^6 to 101^{10} particles) is injected i.m. at day 1 postinjury in the inguinal area of the upper hindlimb following surgery. Alternatively, the full length or soluble n-terminal Shh adenoassociated virus (AAV) or a control LacZ AAV is administered 4 weeks prior to surgery. Similar doses of adenovirus containing full length or n-terminal Shh or LacZ containing control adenovirus can be administered in place of AAV-Shh. Above endpoints for vascular and motor neuron conduction improvements are also seen with viral gene therapy.

Together these results show that the hedgehog pathway is a crucial component of the normal angiogenic response, tissue regeneration and recovery from ischemia injury and that hedgehog proteins can induce angiogenesis and improve recovery from ischemia when used pharmacologically.

Example 6: Hedgehog Induces Collateral Vessel formation and Improved Myocardial Function following Surgically Induced Myocardial Ischemia

Chronic myocardial ischemia and collateral vessel formation can be modeled in pigs through the placement of an ameroid constrictor around the left circumflex coronary artery. Treatment of these ischemic hearts with angiogenic proteins can increase myocardial vascularity, perfusion and function in the ischemic area as well as overall heart function. We determine that hedgehog protein or gene therapy can also

improve these measures of cardiac perfusion, viability and function following ischemia in the following experiments.

Ameroid constrictors are placed around the left circumflex coronary artery (LCX) of anesthetized Yorkshire pigs (5-6 weeks old, 15-18kg, male or female) (Laham et al., 2000; Harada et al., 1994; Unger et al., 1994). The animals are allowed to recover for 3 weeks to allow time for ameroid closure. Either immediately after or 3 weeks post-ameroid placement, the animals are randomized into one of several groups (10 animals/group). Hedgehog or control is administered by one of the following routes:

1. direct injection of ischemic myocardium with hedgehog or saline
2. intrapericardial administration of hedgehog protein or saline
3. systemic administration of hedgehog protein or saline (s.c., i.m. or i.v. injection)
4. myocardial injection of hedgehog in (0.1-5mg) heparin or heparin alone following thoracotomy or via an injection catheter (Cordis-Webster)
5. intrapericardial injection of hedgehog in (0.1-5mg) heparin
6. intracoronary catheter delivery device
7. viral gene therapy via above methods using 10^6-10^{12} Particles of full length or n-terminal Shh adenovirus in a single or several bolus injections (0.lml-lml/injection).

Heart muscle perfusion and function are monitored using several techniques immediately prior to the Hedgehog treatments and 2-4 weeks post-Hedgehog treatments. Coronary perfusion was determined by right and left coronary angiography.

To obtain a collateral index, left to left and right to left coronary collaterals are measured. Regional resting myocardial blood flow is measured using colored microspheres. Magnetic resonance imaging of wall thickening is used to determine global ventricular, ischemic/normal regional function and myocardial perfusion. Electromechanical left ventricular mapping is done using the NOGA system (Biosense, Johnson&Johnson, Warren, NJ) to determine localized heart function (Vale et al., 1999, Kornowski, Hong and Leon, 1998). In addition, complete autopsies and histopathology is done on each animal for coronary tissues (pericardium, epicardial coronary artery, myocardium in the left anterior descending artery distribution (normal tissue), left circumflex artery distribution, (ischemic tissue) and peripheral organs (gastrointestinal

tract, lung, liver, kidney, bone, bone marrow)). Improvements in heart muscle perfusion and function as well as histological analysis of coronary tissue vascularization are assessed. Hedgehog treatments can show improvement in these parameters when compared to control treatments suggesting therapeutic utility for hedgehog treatments in myocardial infarction and coronary artery disease.

Example 7: Inhibition of Hedgehog (Anti-hedgehog blocking antibody) Decreases Tumor Growth Rate and/or Tumor Angiogenesis

To determine if tumor cell lines overexpress hedgehog protein, anti-hedgehog antibody was used to immunoprecipitate cell lysates of various tumor cell lines. We used gastrointestinal epithelial cell lines as an example: T84 (human colon epithelial carcinoma, CCL-284, ATCC, Manassas, VA); Caco2 and SW480 (human colon epithelial adenocarcinomas, HTB-37 and CCL-228, ATCC, Manassas, VA). Briefly, one milligram amounts of cell lysis supernatant were immunoprecipitated with either anti-hedgehog antibody, 5E1 (+) or an isotype matched control antibody, 9E10 (C). The immunoprecipitated samples were analysed by western blotting with an anti-hedgehog rabbit polyclonal antibody, r1200.

More specifically, confluent monolayers of each cell line in T 150 flasks were lysed in 3mL of cold lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.% SDS, 150mM NaCl, 1mM sodium vanadate, 10% glycerol, lOmM Tris-HCL, pH 8.0) containing a 2x concentration of Complete protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN). The lysate was rocked for 30' at 4°C then scraped into a microfuge tube and debris pelleted in a microfuge for 10'. The supernatant was stored at -80°C. Protein concentration of the supernatants were determined using Bio-Rad Protein Assay reagent and equivalent milligram amounts of supernatant were used for each immunoprecipitation. Each sample was gently agitated overnight at 4°C with 2.5 ug of either anti-hedgehog antibody, 5E1, or an isotype matched control antibody, 9E10 (anti-human c-myc, Calbiochem, San Diego, CA) (Fan et al., 1998). Protein A conjugated Sepharose beads (30 microliters packed beads/sample) were added to each sample and the samples were gently agitated at 4°C for 30-40 minutes. The beads and associated

immune complexes were then spun down in a microfuge for 10 seconds and washed 4 times with 1 ml of ice cold lysis buffer. The buffer was then removed from the beads, reducing SDS-PAGE sample buffer was added, the samples were heated to 90°C for 5 minutes then analyzed by SDS-PAGE (4-20% Tris-glycine gels, Novex, San Diego, CA). The proteins were transferred to nitrocellulose filters and western blot analysis was performed at room temperature.

The nitrocellulose filters was incubated with blocking solution (5% dry milk in Tris-buffered saline with 0.3% Tween-20) for 1 hour followed by blocking solution containing a 1:10,000 dilution of anti-hedgehog rabbit polyclonal, r1200, for 2-3 hours at room temperature or overnight incubation at 4°C. The nitrocellulose filters were washed 3 times with Tris-buffered saline with 0.3% Tween-20; incubated for 1 hour in 1:5000 dilution of horseradish peroxidase-conjugated goat anti-rabbit antibody (Jackson Immunoresearch) then visualized using ECL western blotting detection reagents (Amersham Pharmacia Biotech).

Hedgehog protein is overexpressed in several human gastrointestinal tumor cell lines compared to normal human gastrointestinal epithelial cells or fibroblasts (data not shown). The anti-hedgehog antibody immunoprecipitations show a hedgehog rabbit polyclonal antibody-reactive band at 19kD, the expected molecular weight for hedgehog protein. The control antibody (9E10) immunoprecipitation shows no hedgehog polyclonal antibody-reactive band comigrating with hedgehog protein standard at 19kD. Normal gastrointestinal epithelial also express a low level of hedgehog protein, but normal gastrointestinal fibroblasts do not show any expression. None of the epithelial cell lines tested respond to hedgehog (data not shown), but the hedgehog produced by these tumor cells may activate angiogenesis via induction of stromal tissue in the tumor.

The ability of hedgehog-blocking or hedgehog pathway-blocking reagents such as the anti-hedgehog blocking antibodies (5E1, ARG6, ALC9 or BH.E4) to inhibit tumor angiogenesis and tumor growth are determined in subcutaneously-implanted tumor models in athymic Swiss (Cr:NIH(S)-nu) or athymic random bred (NCr-nu) mice of a single sex (males >18g or females > 17g, all within a 4g weight range). Carcinoma cell lines of gastrointestinal origin such as SW480, HT29 or T84 are passaged in nude

mice as subcutaneous tumors or are passsaged in culture as cell monolayers. Either 2x 10^6 cells or tumor 20-40mg fragments of a passaged tumor are implanted subcutaneously in the axillary region of 6-10 athymic mice. Tumors were monitored frequently for progressive growth. Treatments are initiated when individual tumors range between 100 mg - 700 mg. Mice are randomized into test and vehicle control groups and treated with either hedgehog blocking antibodies, control isotope-matched antibody, no treatement or cisplatinum. Antibodies were administered (25-100mg/kg bolus i.p. injections) at a frequency of every day to 3 times a week for the follow-up period. Cisplatinum was administered subcutaneously three times a week (2 mg/kg). Body weights and tumor measurements (width and length) are recorded at 3 - 5 day intervals following treatment for 7-21 days. Tumors are collected on the final day for histological analysis. Mean tumor weight change and/or mean vascular density are decreased in the hedgehog blocking antibody-treated group compared to the control antibody-treated group. In addition, hedgehog blocking antibodies may be administered prior to tumor implantation and tumor growth rate is monitored as described to determine if early tumor growth rates are decreased by blocking hedgehog signalling.

Example 8: Production and Expression of HH-Ig fusions

MATERIALS AND METHODS

Construction of pUB55, expression plasmid for Sonic Hedgehog in Pichia pastoris:

pUB55 contains the N-terminal domain of human Sonic Hedgehog (SEQ ID NO: 21 in Table 4) with the alpha factor PrePro region as the secretion signal. pUB55 was constructed in pCCM73, a derivative of pPIC9 (obtained from Invitrogen, San Diego, CA) with the Kanamycin gene (HincII-HincII fragment) of pUC4-K inserted at the SphI site of pPIC9. The human Sonic hedgehog coding sequence from EarI-NotI was obtained from pEAG543 which has a stop codon and Not 1 site engineered following Gly197 in the coding sequence. Plasmid pCCM73 was cut with XhoI and NotI and was ligated with the EarI-NotI fragment of pEAG543 (containing the Sonic Hedgehog coding sequence, Table 4) and oligonucleotides [5' TCG AGA AAA GAT GCG GAC CGG GCA GGG GGT 3': SEQ ID NO: 36 and 5' CGA ACC CCC TGC CCG GTC

CGC ATC TTT TC 3': SEQ ID NO: 37] that form a XhoI-EarI fragment and create the appropriate coding sequence for placing Sonic hedgehog adjacent to the alpha factor leader sequence in frame.

Expression of Desert Hedgehog in Pichia pastoris and construction of KEX2 site mutations:

The Desert Hedgehog coding region in plasmid pEAG680 was modified to incorporate a BsrGI and an XmaI site site using the Stratagene QuikChange mutagenesis kit.

Expression of Indian Hedgehog in Pichia pastoris and construction of KEX2 site mutattions:

Plasmid pEAG657 is pBluescript with the Indian Hedgehog coding sequence with a stop codon following codon GlyXXX. pEAG658 is pBluescript with the Indian Hedgehog coding sequence and a SalI site engineered within residues suitable for fusing the Indian Hedgehog coding sequence (SEQ ID NO: 22) with Fc immunoglobulin coding sequences (SEQ ID NOS: 28-30) at the hinge region of immunoglobulins. To facilitate subsequent manipulations, SpeI and XmaI sites were introduced to pEAG658 by site-directed mutagenesis.

Table: DNA sequences of Hedgehog N-terminal domains and Immunoglobulin Fc Regions:

Protein	DNA Sequence
Human Sonic Hedgehog N-	TGCGGACCGGGCAGGGGGTTCGGGAAGAGGAGG
terminal Domain	CACCCCAAAAAGCTGACCCCTTTAGCCTACAAGC

AGTTTATCCCCAATGTGGCCGAGAAGACCCTAG

[SEQ ID NO: 21] GCGCCAGCGGAAGGTATGAAGGGAAGATCTCCA

GAAACTCCGAGCGATTTAAGGAACTCACCCCCA

ATTACAACCCCGACATCATATTTAAGGATGAAG

AAAACACCGGAGCGGACAGGCTGATGACTCAGA

GGTGTAAGGACAAGTTGAACGCTTTGGCCATCTC

GGTGATGAACCAGTGGCCAGGAGTGAAACTGCG

GGTGACCGAGGGCTGGGACGAAGATGGCCACCA

CTCAGAGGAGTCTCTGCACTACGAGGGCCGCGC

AGTGGACATCACCACGTCTGACCGCGACCGCAG

CAAGTACGGCATGCTGGCCCGCCTGGCGGTGGA

GGCCGGCTTCGACTGGGTGTACTACGAGTCCAA

GGCACATATCCACTGCTCGGTGAAAGCAGAGAA

CTCGGTGGCGGCCAAATCGGGAGGC

Human Indian Hedgehog TGCGGGCCGGGTCGGGTGGTGGGCAGCCGCCGG

N-terminal Domain	CGACCGCCACGCAAACTCGTGCCGCTCGCCTACA
	AGCAGTTCAGCCCCAATGTGCCCGAGAAGACCC
[SEQ ID NO: 22]	TGGGCGCCAGCGGACGCTATGAAGGCAAGATCG
	CTCGCAGCTCCGAGCGCTTCAAGGAGCTCACCCC
	CAATTACAATCCAGACATCATCTTCAAGGACGA
	GGAGAACACAGGCGCCGACCGCCTCATGACCCA
	GCGCTGCAAGGACCGCCTGAACTCGCTGGCTATC
	TCGGTGATGAACCAGTGGCCCGGTGTGAAGCTG
	CGGGTGACCGAGGGCTGGGACGAGGACGGCCAC
	CACTCAGAGGAGTCCCTGCATTATGAGGGCCGC
	GCGGTGGACATCACCACATCAGACCGCGACCGC
	AATAAGTATGGACTGCTGGCGCGCTTGGCAGTG
	GAGGCCGGCTTTGACTGGGTGTATTACGAGTCAA
	AGGCCCACGTGCATTGCTCCGTCAAGTCCGAGCA
	CTCGGCCGCAGCCAAGACGGGCGGC

Human Desert Hedgehog	TGCGGGCCGGGCCGGGGGCCGGTTGGCCGGCGC
N-terminal Domain	CGCTATGCGCGCAAGCAGCTCGTGCCGCTACTCT
	ACAAGCAATTTGTGCCCGGCGTGCCAGAGCGGA
	CCCTGGGCGCCAGTGGGCCAGCGGAGGGGAGGG
[SEQ ID NO: 27]	TGGCAAGGGGCTCCGAGCGCTTCCGGGACCTCG
	TGCCCAACTACAACCCCGACATCATCTTCAAGGA
	TGAGGAGAACAGTGGAGCCGACCGCCTGATGAC
	CGAGCGTTGTAAGGAGCGGGTGAACGCTTTGGC
	CATTGCCGTGATGAACATGTGGCCCGGAGTGCG
	CCTACGAGTGACTGAGGGCTGGGACGAGGACGG
	CCACCACGCTCAGGATTCACTCCACTACGAAGGC
	CGTGCTTTGGACATCACTACGTCTGACCGCGACC
	GCAACAAGTATGGGTTGCTGGCGCGCCTCGCAG
	TGGAAGCCGGCTTCGACTGGGTCTACTACGAGTC
	CCGCAACCACGTCCACGTGTCGGTCAAAGCTGAT
	AACTCACTGGCGGTCCGGGCGGGCGGC
Fc region of human IgGI--	GTCGACAAAACTCACACATGCCCACCGTGCCCA
with Asn-Gln glycosylation	GCACCTGAACTCCTGGGGGGACCGTCAGTCTTCC
site mutation	TCTTCCCCCCAAAACCCAAGGACACCCTCATGAT

CTCCCGGACCCCTGAGGTCACATGCGTGGTGGTG
GACGTGAGCCACGAAGACCCTGAGGTCAAGTTC
[SEQ ID. NO: 28] AACTGGTACGTGGACGGCGTGGAGGTGCATAAT
GCCAAGACAAAGCCGcgggaggagcagtaccagagcacgtacc
gtgtggTCAGCGTCCTCACCGTCCTGCACCAGGACT
GGCTGAATGGCAAGGAGTACAAGTGCAAGGTCT
CCAACAAAGCCCTCCCAGCCCCCATCGAGAAAA
CCATCTCCAAAGCCAAAGGGCAGCCCCGAGAAC
CACAGGTGTACACCCTGCCCCCATCCCGGGATGA
GCTGACCAAGAACCAGGTCAGCCTGACCTGCCT
GGTCAAAGGCTTCTATCCCAGCGACATCGCCGTG
GAGTGGGAGAGCAATGGGCAGCCGGAGAACAA
CTACAAGACCACGCCTCCCGTGTTGGACTCCGAC
GGCTCCTTCTTCCTCTACAGCAAGCTCACCGTGG
ACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCT

	CATGCTCCGTGATGCATGAGGCTCTGCACAACCA
	CTACACGCAGAAGAGCCTCTCCCTGTCTCCCGGG
	AAA
Fc region of murine IgGl--	GTCGACGTGCCCAGGGATTGTGGTTGTAAGCCTT
with Asn-Gln glycosylation	GCATATGTACAGTCCCAGAAGTATCATCTGTCTT
site mutation	CATCTTCCCCCCAAAGCCCAAGGATGTGCTCACC
	ATTACTCTGACTCCTAAGGTCACGTGTGTTGTGG
	TAGACATCAGCAAGGATGATCCCGAGGTCCAGT
[SEQ ID NO: 29]	TCAGCTGGTTTGTAGATGATGTGGAGGTGCACAC
	AGCTCAGACGCAACCaCGGGAaGAGCAGTTCCAA
	AGCACTTTCCGCTCAGTCAGTGAACTTCCCATCA
	TGCACCAGGACTGGCTCAATGGCAAGGAGTTCA
	AATGCAGGGTCAACAGTGCAGCTTTCCCTGCCCC
	CATCGAGAAAACCATCTCCAAAACCAAAGGCAG
	ACCGAAGGCTCCACAGGTGTACACCATTCCACCT

	CCCAAGGAGCAGATGGCCAAGGATAAAGTCAGT CTGACCTGCATGATAACAGACTTCTTCCCTGAAG ACATTACTGTGGAGTGGCAGTGGAATGGGCAGC CAGCGGAGAACTACAAGAACACTCAGCCCATCA TGGACACAGATGGCTCTTACTTCGTCTACAGCAA GCTCAATGTGCAGAAGAGCAACTGGGAGGCAGG AAATACTTTCACCTGCTCTGTGTTACATGAGGGC CTGCACAACCACCATACTGAGAAGAGCCTCTCCC ACTCTCCTGGTAAA
Fc region of murine IgG2a-- with Asn-Gln glycosylation site mutation [SEQ ID NO: 30]	GTCGACCCCAGAGGGCCCACAATCAAGCCCTGT CCTCCATGCAAATGCCCAGCACCTAACCTCTTGG GTGGACCATCCGTCTTCATCTTCCCTCCAAAGAT CAAGGATGTACTCATGATCTCCCTGAGCCCCATA GTCACATGTGTGGTGGTGGATGTGAGCGAGGAT GACCCAGATGTCCAGATCAGCTGGTTTGTGAACA ACGTGGAAGTACACACAGCTCAGACACAAACCC ATAGAGAGGATTACCAAAGTACaCTtCGGGTGGT CAGTGCCCTCCCCATCCAGCACCAGGACTGGATG

AGTGGCAAGGAGTTCAAATGCAAGGTCAACAAC

AAAGACCTCCCAGCGCCCATCGAGAGAACCATC

TCAAAACCCAAAGGGTCAGTAAGAGCTCCACAG

GTATATGTCTTGCCTCCACCAGAAGAAGAGATG

ACTAAGAAACAGGTCACTCTGACCTGCATGGTG

ACAGACTTCATGCCTGAAGACATTTACGTGGAGT

GGACCAACAACGGGAAAACAGAGCTAAACTACA

AGAACACTGAACCAGTCCTGGACTCTGATGGTTC

TTACTTCATGTACAGCAAGCTGAGAGTGGAAAA

GAAGAACTGGGTGGAAAGAAATAGCTACTCCTG

TTCAGTGGTCCACGAGGGTCTGCACAATCACCAC

ACGACTAAGAGCTTCTCCCGGACTCCGGGTAAA

Plasmid DNA sequence

PUB55

GATCTAACATCCAAAGACGAAAGGTTGAATGAAACCTTTTTGCCAT

CCGACATCCACAGGTCCATTCTCACACATAAGTGCCAAACGCA
AC
SEQID
AGGAGGGGATACACTAGCAGCAGACCGTTGCAAACGCAGGACCTC
N0:31
CACTCCTCTTCTCCTCAACACCCACTTTTGCCATCGAAAAACCAGC
CCAGTTATTGGGCTTGATTGGAGCTCGCTCATTCCAATTCCTTC
TAT
TAGGCTACTAACACCATGACTTTATTAGCCTGTCTATCCTGGCC
CC
CCTGGCGAGGTTCATGTTTGTTTATTTCCGAATGCAACAAGCT
CCG
CATTACACCCGAACATCACTCCAGATGAGGGCTTTCTGAGTGT
GGG
GTCAAATAGTTTCATGTTCCCCAAATGGCCCAAAACTGACAGT
TTA
AACGCTGTCTTGGAACCTAATATGACAAAAGCGTGATCTCATC
CAA
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AA TAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCAC

Construction of Hedgehog-Ig Fusion Proteins

Shh-Fc(muIgGl) plasmid pUB114 (SEQ ID NO: 32), has the wild-type SHH domain (SEQ ID NO: 21 or 23) fused to the CH2 and CH3 regions of murine IgGI (SEQ ID NO: 29).

The Fc region in pUBl14 contains a glycosylation site mutation [Asn297G1n]. Plasmid pUB55 (SEQ ID NO: 31) and pUB 114 plasmids are identical outside of the region coding for the Fc domain fused to SHH. Plasmids identical to pUB 114, but containing the human IgGI or murine IgG2a Fc region are pUB115 (SEQ ID NO: 33) and pUB 116 (SEQ ID NO: 34), respectively.

For construction of yeast strains expressing protein, plasmids were digested with Stul and transformed into Pichia pastoris GS115 by electroporation in 1M Sorbitol (Invitrogen) or by a Li salt transformation procedure (Frozen EZ Yeast Transformation kit, Zymo Research, Orange, CA). His+ transformants were selected on MD agar. Colonies were purified on YPD agar and cultured for protein expression in 5 ml BMMY (2% Methanol) medium. BMMY culture supernatants were harvested at 1 or 2 days (1-day harvests were concentrated by TCA precipitation) and were analyzed by SDS-PAGE and Coomassie blue staining to distinguish clipped and unclipped SHE

Protein Purification

Large scale preparations of protein for purification were prepared as follows: An inoculum in BMGY (late log to stationary phase) was added to 1 L BMGY in a Fernbach flask and incubated at 150 rpm for 2-3 days. The stationary phase BMGY culture was centrifuged and the cell pellet from 1 L was resuspended in BMMY(2% Methanol) and incubated in a Fernbach flask at 30 C for 2-3 days. Pepstatin A (44 microM) was added to BMMY medium for expression of SHH-Fc fusion proteins.

A. Purification of Hedgehog-Ig fusion protein constructs

Pichia cells were removed from the conditioned medium by centrifugation before application to Protein A Fast Flow ® (Pharmacia). Protein from constructs utilizing human IgGI (SEQ ID NO: 28) or murine IgG2A sequences (SEQ ID NO: 30) were applied directly to the Protein A. Constructs utilizing murine IgG 1 sequences were diluted ten-fold with water to reduce the salt concentration, re-concentrated using a 3K cutoff spiral filter (Amicon) and the pH adjusted with the addition of sodium borate buffer, pH 8.5 to a final concentration of 50 mM.

HHIg was eluted with 25 mM sodium phosphate, pH 2.8, and the fractions collected into tubes containing 0.1 volume of 0.5 M sodium phosphate pH 6 to readjust the pH. The Protein A eluant was then diluted eight-fold with 0.5 mM sodium phosphate, pH 6 and applied to a CM-Poros® column (Perseptive Biosystems) equilibrated with 50 mM sodium phosphate, pH 6.0. Elution with a gradient of 0-0.8 M NaCI separated two HHIg peaks.

The first is "one-armed" protein in which one of the HHIg polypeptides of the dimer is proteolytically cleaved at a sequence near the hinge and therefore this dimer contains only one HH N-terminal domain. The second peak is the dimer with two full-length HHIg chains. The peaks were pooled separately, reduced with 10 mM DTT and dialyzed against 5 mM sodium phosphate, pH 5.5, 150 mM NaCI and 0.5 mM DTT. No DTT was used when the N-terminal cysteine of the protein was replaced with other amino acids. These two purification steps achieve >95% purity. Purity was determined by SDS-PAGE on 4-20% gradient gels (Novex) stained with Coomassie Blue. Identity

was confirmed by mass spectrometry, and potency was analyzed using a cell-based bioactivity assay (see above).

Mass spectrometry

The molecular masses of the purified proteins were determined by electrospray ionization mass spectroscopy (ESI-MS) on a Micromass Quattro II triple quadrupole mass spectrometer. Samples were desalted using an on-line Michrom Ultrafast Microprotein Analyzer system with a Reliasil© C4 column (1 mm x 5 cm). All electrospray mass spectral data were processed using the Micromass MassLynx data system.

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119

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We claim:

1. A method of promoting angiogenesis in a subject animal comprising administering to the subject an angiogenic amount of a hedgehog polypeptide or agonist thereof.

2. The method of claim 1, wherein the step of administering comprises contacting the hedgehog polypeptide or agonist with a mesenchymal cell of the subject.

3. The method of claim 1, comprising administering to the subject a polypeptide including a hedgehog amino acid sequence, which hedgehog sequence directs the binding of the polypeptide to a patched receptor polypeptide and/or induces alkaline phosphatase activity in C3H10T1/2 cells.

4. The method of claim 1, comprising administering to the subject a polypeptide including a hedgehog amino acid sequence having at least 60% amino acid identity with SEQ ID No. 10-18 or 20.

5. The method of claim 1, comprising administering to the subject a polypeptide including a hedgehog amino acid sequence encoded by a coding sequence which hybridizes under stringent conditions to any of SEQ ID No. 1-9 or 19.

6. The method of claim 1, comprising administering to the subject a polypeptide including a hedgehog amino acid sequence represented by SEQ ID No. 26.

7. The method of any of claims 3 – 7, wherein the hedgehog sequence includes at least 50 resdiues of an extracellular domain of a hedgehog protein.

8. The method of any of claims 3 – 7, wherein the polypeptide is derivatized with one or more chemical moieties.

9. The method of claim 8, wherein the chemical moiety is a polyalkylene glycol polymer.

10. The method of claim 8, wherein the chemical moiety is a hydrophobic moiety.

11. The method of claim 10, wherein the hydrophobic moiety is a lipid.

12. The method of claim 8, wherein the chemical moiety is one or more phosphate groups.

13. The method of claim 8, wherein the chemical moiety is one or more acetyl groups.

14. The method of claim 8, wherein the chemical moiety is one or more sugar or carbohydrate groups.

15. The method of claim 8, wherein the chemical moieties are any combination of phosphate, acetyl, sugar, carbohydrate, or hydrophobic moieties.

16. The method of claim 4, wherein the method further comprises administering an agent that enhances agonistic properties of the hedgehog therapeutic.

17. The method of claim 16, wherein the agent is an angiogenic factor selected from the group consisting of vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), angiopoietin 1, angiopoietin 2, monocyte chemotactic protein-1 (MCP-1).

18. A method of inhibiting angiogenesis in a subject animal comprising administering to the subject an antiangiogenic amount of a hedgehog antagonist.

19. The method of claim 18, comprising administering a polypeptide including one or more antigen binding domains which bind to and inhibit hedgehog signalling.

20. The method of claim 18, comprising administering a polypeptide including one or more antigen binding domains which bind to patched and inhibit hedgehog signalling.

21. The method of claim 18, comprising administering a polypeptide including one or more antigen binding domains which bind to smoothened and inhibit hedgehog signalling.

22. The method of claim 19, 20 or 21, wherein the antigen binding domain is part of a an antibody structure selected from the group consisting of a humanized antibody homology, a human antibody homolog, a chimeric antibody homolog and fragments thereof.

23. The method of claim 18, comprising administering a functional antagonist of a hedgehog therapeutic.

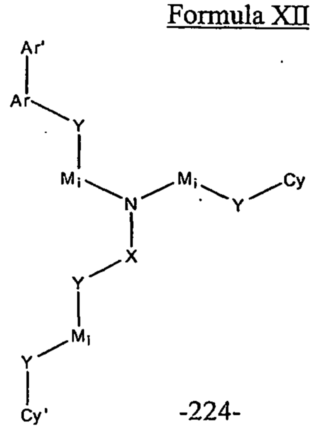
24. The method of claim 18, or 20, wherein the subject has a condition selected from the group consisting of a malignant tumor, retinopathy, macular degeneration, a nonmalignant tumor, rheumatoid arthritis, osteoarthritis, neovascular glaucoma, keloids, Crohn's disease, ulcerative colitis, and psoriasis.

25. The method of claim 1, wherein the hedgehog agonist is a small organic molecule.

26. The method of claim 25, wherein the hedgehog agonist has a molecular weight less than 2500 amu.

27. The method of claim 25, wherein the hedgehog agonist is represented by general formula (XII):

Formula XII



wherein, as valence and stability permit,

Ar and Ar' independently represent substituted or unsubstituted aryl or heteroaryl rings;

Y, independently for each occurrence, may be absent or represent -N(R)-, -O-, -S-, or -Se-;

X can be selected from -C(=O)-, -C(=S)-, -S(O_2)-, -S(O)-, -C(=NCN)-, -P(=O)(OR_2)-, and a methylene group optionally substituted with 1-2 groups such as lower alkyl, alkenyl, or alkynyl groups;

M represents, independently for each occurrence, a substituted or unsubstituted methylene group, such as -CH_2-, -CHF-, -CHOH-, -CH(Me)-, -C(=O)-, etc., or two M taken together represent substituted or unsubstituted ethene or ethyne;

R represents, independently for each occurrence, H or substituted or unsubstituted aryl, heterocyclyl, heteroaryl, aralkyl, heteroaralkyl, alkynyl, alkenyl, or alkyl, or two R taken together may form a 4- to 8-membered ring, e.g., with N;

Cy and Cy' independenly represent substituted or unsubstituted aryl, heterocyclyl, heteroaryl, or cycloalkyl, including polycyclic groups;

i represents, independently for each occurrence, an integer from 0 to 5, preferably from 0 to 2; and

n, individually for each occurence, represents an integer from 0 to 10, preferably from 0 to 5.

28. The method of any of claims 3 – 7, comprising administering a nucleic acid sequence encoding the polypeptide.

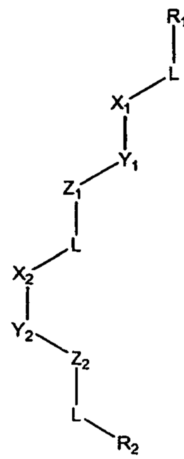
29. The method of claim 29, wherein the nucleic acid sequences encoding the polypeptide are introduced via a viral vector, via lipofection, and/or as naked DNA.

30. The method of claim 18, wherein the hedgehog antagonist is a small organic molecule.

31. The method of claim 30, wherein the hedgehog antagonist has a molecular weight less than 2500 amu.

32. The method of claim 30, wherein the hedgehog antagonist is represented by one or more of formulas I – XI.

33. The method of claim 30, wherein the hedgehog antagonist is represented by general formula (I):



wherein, as valence and stability permit,

R_1 and R_2, independently for each occurrence, represent H, lower alkyl, aryl (e.g., substituted or unsubstituted), aralkyl (e.g., substituted or unsubstituted, e.g., $-(CH_2)_n$aryl), or heteroaryl (e.g., substituted or unsubstituted), or heteroaralkyl (e.g., substituted or unsubstituted, e.g., $-(CH_2)_n$heteroaralkyl-);

L, independently for each occurrence, is absent or represents $-(CH_2)_n$-alkyl, -alkenyl-, -alkynyl-, $-(CH_2)_n$alkenyl-, $-(CH_2)_n$alkynyl-, $-(CH_2)_nO(CH_2)_p$-, $-(CH_2)_nNR_2(CH_2)_p$-, $-(CH_2)_nS(CH_2)_p$-, $-(CH_2)_n$alkenyl$(CH_2)_p$-, $-(CH_2)_n$alkynyl$(CH_2)_p$-, $-O(CH_2)_n$-, $-NR_2(CH_2)_n$-, or $-S(CH_2)_n$-;

X_1 and X_2 can be selected, independently, from $-N(R_8)$-, -O-, -S-, -Se-, -N=N-, -ON=CH-, $-(R_8)N-N(R_8)$-, $-ON(R_8)$-, a heterocycle, or a direct bond between L and Y_1 or Y_2, respectively;

Y_1 and Y_2 can be selected, independently, from -C(=O)-, -C(=S)-, $-S(O_2)$-, -S(O)-, -C(=NCN)-, $-P(=O)(OR_2)$-, a heteroaromatic group, or a direct bond between X_1 and Z_1 or X_2 and Z_2, respectively;

Z_1 and Z_2 can be selected, independently, from $-N(R_8)$-, -O-, -S-, -Se-, -N=N-, -ON=CH-, $-R_8N-NR_8$-, $-ONR_8$-, a heterocycle, or a direct bond between Y_1 or Y_2, respectively, and L;

R_8, independently for each occurrence, represents H, lower alkyl, $-(CH_2)_n$aryl (e.g., substituted or unsubstituted), $-(CH_2)_n$heteroaryl (e.g., substituted or unsubstituted), or two R_8 taken together may form a 4- to 8-membered ring, e.g., with X_1 and Z_1 or X_2 and Z_1, which ring may include one or more carbonyls;

p represents, independently for each occurrence, an integer from 0 to 10, preferably from 0 to 3; and

n, individually for each occurence, represents an integer from 0 to 10, preferably from 0 to 5.

Figure 1 : Alignment of N-terminal fragments of Human Hedgehog Proteins

	1					
Indian	CGPGRVVGSR	RRPPRK-LVP	LAYKQFSPNV	PEKTLGASGR	YEGKIARSSE	
Sonic	CGPGRGFG-K	RRHPKK-LTP	LAYKQFIPNV	AEKTLGASGR	YEGKISRNSE	
Desert	CGPGRGPVGR	RRYARKQLVP	LLYKQFVPGV	PERTLGASGP	AEGRVARGSE	
	51					
Indian	RFKELTPNYN	PDIIFKDEEN	TGADRLMTQR	CKDRLNSLAI	SVMNQWPGVK	
Sonic	RFKELTPNYN	PDIIFKDEEN	TGADRLMTQR	CKDKLNALAI	SVMNQWPGVK	
Desert	RFRDLVPNYN	PDIIFKDEEN	SGADRLMTER	CKERVNALAI	AVMNMWPGVR	
	101					
Indian	LRVTEGWDED	GHHSEESLHY	EGRAVDITTS	DRDRNKYGLL	ARLAVEAGFD	
Sonic	LRVTEGWDED	GHHSEESLHY	EGRAVDITTS	DRDRSKYGML	ARLAVEAGFD	
Desert	LRVTEGWDED	GHHAQDSLHY	EGRALDITTS	DRDRNKYGLL	ARLAVEAGFD	
	151					
Indian	WVYYESKAHV	HCSVKSEHSA	AAKTGG	SEQ ID NO: 23		
Sonic	WVYYESKAHI	HCSVKAENSV	AAKSGG	SEQ ID NO. 24		
Desert	WVYYESRNHV	HVSVKADNSL	AVRAGG	SEQ ID NO. 25		

Gap(s), indicated by -, added to facilitate alignment

Figure 2: SEQ ID NO: 26 is the consensus sequence of a hedgehog protein suitable for use in developing the conjugated proteins of the invention, antagonist, where "Xaa" indicates amino acids that differ between the Sonic, Indian and Desert hedgehog proteins.

C* G P G R Xaa1 Xaa2 Xaa3 Xaa4 Xaa5 R R Xaa6 Xaa7 Xaa8 K Xaa9 L Xaa10 P

L Xaa11 Y K Q F Xaa12 P Xaa13 V Xaa14 E K T L G A S G R

Xaa15 E G K Xaa16 Xaa17 R Xaa18 S E R F K Xaa19 L Xaa20 P N Y N

P D I I F K D E E N Xaa21 G A D R L M T Xaa22 R

C K Xaa23 Xaa24 Xaa25 N S L A I Xaa26 V M N Xaa27 W P G V K

L R V T E G W D E D G H H X2aa8 Xaa29 Xaa30 S L H Y
E G R A V D I T T S D R D R Xaa31 K Y G Xaa32 L

A R L A V E A G F D W V Y Y E S Xaa33 Xaa34 H Xaa35

H Xaa36 S V K Xaa37 Xaa38 Xaa39 S Xaa40 A A Xaa41 Xaa42 G G

Where
C* is a cysteine that may be modified, altered or substituted within another moiety or series of moieties as described herein;

Xaa1 is either V or G; Xaa2 is either V, E or P Xaa3 is either G or V
Xaa4 is either S or G; Xaa5 is either R or K; Xaa6 is either P, H or Y;
Xaa7 is either P or A; Xaa8 is either R or K; Xaa9 is any amino acid;
Xaa10 is either V or T; Xaa11 is either A or L;
Xaa12 is either S, I or V; Xaa13 is either N or G; Xaa14 is either P or A;
Xaa15 is either Y or A; Xaa16 is either I or V; Xaa17 is either A or S;
Xaa18 is either S, N or G; Xaa19 is either E or D; Xaa20 is either T or V;
Xaa21 is either T or S; Xaa22 is either Q or E; Xaa23 is either D or E;
Xaa24 is either R or K; Xaa25 is either L or V; Xaa26 is either S or A;
Xaa27 is either Q or M; Xaa28 is either S or A; Xaa29 is either E or Q;
Xaa30 is either E or D; Xaa31 is either N or S; Xaa32 is either L or M;
Xaa33 is either K or R; Xaa34 is either A or N; Xaa35 is either V or I;
Xaa36 is either C or V; Xaa37 is either S or A; Xaa38 is either E or D;
Xaa39 is either H or N; Xaa40 is either A, V or L; Xaa41 is either K or R; and
Xaa42 is either T, S or A.

SEQUENCE LISTING

(2) INFORMATION FOR SEQ ID NO:1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1277 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1275

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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ATG GTC GAA ATG CTG CTG TTG ACA AGA ATT CTC TTG GTG GGC TTC ATC       48
Met Val Glu Met Leu Leu Leu Thr Arg Ile Leu Leu Val Gly Phe Ile
  1               5                  10                  15

TGC GCT CTT TTA GTC TCC TCT GGG CTG ACT TGT GGA CCA GGC AGG GGC       96
Cys Ala Leu Leu Val Ser Ser Gly Leu Thr Cys Gly Pro Gly Arg Gly
             20                  25                  30

ATT GGA AAA AGG AGG CAC CCC AAA AAG CTG ACC CCG TTA GCC TAT AAG      144
Ile Gly Lys Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys
         35                  40                  45

CAG TTT ATT CCC AAT GTG GCA GAG AAG ACC CTA GGG GCC AGT GGA AGA      192
Gln Phe Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg
     50                  55                  60

TAT GAA GGG AAG ATC ACA AGA AAC TCC GAG AGA TTT AAA GAA CTA ACC      240
Tyr Glu Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr
 65                  70                  75                  80

CCA AAT TAC AAC CCT GAC ATT ATT TTT AAG GAT GAA GAG AAC ACG GGA      288
Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly
                 85                  90                  95

GCT GAC AGA CTG ATG ACT CAG CGC TGC AAG GAC AAG CTG AAT GCC CTG      336
Ala Asp Arg Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu
            100                 105                 110

GCG ATC TCG GTG ATG AAC CAG TGG CCC GGG GTG AAG CTG CGG GTG ACC      384
Ala Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg Val Thr
        115                 120                 125

GAG GGC TGG GAC GAG GAT GGC CAT CAC TCC GAG GAA TCG CTG CAC TAC      432
Glu Gly Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr
    130                 135                 140

GAG GGT CGC GCC GTG GAC ATC ACC ACG TCG GAT CGG GAC CGC AGC AAG      480
Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Ser Lys
145                 150                 155                 160
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TAC GGA ATG CTG GCC CGC CTC GCC GTC GAG GCC GGC TTC GAC TGG GTC      528
Tyr Gly Met Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val
                165                 170                 175

TAC TAC GAG TCC AAG GCG CAC ATC CAC TGC TCC GTC AAA GCA GAA AAC      576
Tyr Tyr Glu Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn
            180                 185                 190

TCA GTG GCA GCG AAA TCA GGA GGC TGC TTC CCT GGC TCA GCC ACA GTG      624
Ser Val Ala Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Thr Val
        195                 200                 205

CAC CTG GAG CAT GGA GGC ACC AAG CTG GTG AAG GAC CTG AGC CCT GGG      672
His Leu Glu His Gly Gly Thr Lys Leu Val Lys Asp Leu Ser Pro Gly
    210                 215                 220

GAC CGC GTG CTG GCT GCT GAC GCG GAC GGC CGG CTG CTC TAC AGT GAC      720
Asp Arg Val Leu Ala Ala Asp Ala Asp Gly Arg Leu Leu Tyr Ser Asp
225                 230                 235                 240

TTC CTC ACC TTC CTC GAC CGG ATG GAC AGC TCC CGA AAG CTC TTC TAC      768
Phe Leu Thr Phe Leu Asp Arg Met Asp Ser Ser Arg Lys Leu Phe Tyr
                245                 250                 255

GTC ATC GAG ACG CGG CAG CCC CGG GCC CGG CTG CTA CTG ACG GCG GCC      816
Val Ile Glu Thr Arg Gln Pro Arg Ala Arg Leu Leu Leu Thr Ala Ala
            260                 265                 270

CAC CTG CTC TTT GTG GCC CCC CAG CAC AAC CAG TCG GAG GCC ACA GGG      864
His Leu Leu Phe Val Ala Pro Gln His Asn Gln Ser Glu Ala Thr Gly
        275                 280                 285

TCC ACC AGT GGC CAG GCG CTC TTC GCC AGC AAC GTG AAG CCT GGC CAA      912
Ser Thr Ser Gly Gln Ala Leu Phe Ala Ser Asn Val Lys Pro Gly Gln
    290                 295                 300

CGT GTC TAT GTG CTG GGC GAG GGC GGG CAG CAG CTG CTG CCG GCG TCT      960
Arg Val Tyr Val Leu Gly Glu Gly Gly Gln Gln Leu Leu Pro Ala Ser
305                 310                 315                 320

GTC CAC AGC GTC TCA TTG CGG GAG GAG GCG TCC GGA GCC TAC GCC CCA     1008
Val His Ser Val Ser Leu Arg Glu Glu Ala Ser Gly Ala Tyr Ala Pro
                325                 330                 335

CTC ACC GCC CAG GGC ACC ATC CTC ATC AAC CGG GTG TTG GCC TCC TGC     1056
Leu Thr Ala Gln Gly Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys
            340                 345                 350

TAC GCC GTC ATC GAG GAG CAC AGT TGG GCC CAT TGG GCC TTC GCA CCA     1104
Tyr Ala Val Ile Glu Glu His Ser Trp Ala His Trp Ala Phe Ala Pro
        355                 360                 365

TTC CGC TTG GCT CAG GGG CTG CTG GCC GCC CTC TGC CCA GAT GGG GCC     1152
Phe Arg Leu Ala Gln Gly Leu Leu Ala Ala Leu Cys Pro Asp Gly Ala
    370                 375                 380

ATC CCT ACT GCC GCC ACC ACC ACC ACT GGC ATC CAT TGG TAC TCA CGG     1200
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385                 390                 395                 400
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CTC CTC TAC CGC ATC GGC AGC TGG GTG CTG GAT GGT GAC GCG CTG CAT      1248
Leu Leu Tyr Arg Ile Gly Ser Trp Val Leu Asp Gly Asp Ala Leu His
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CCG CTG GGC ATG GTG GCA CCG GCC AGC TG                               1277
Pro Leu Gly Met Val Ala Pro Ala Ser
            420                 425
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1190 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1191

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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  1               5                  10                  15

GCA CTA TCT GCC CAG AGC TGC GGG CCG GGC CGA GGA CCG GTT GGC CGG        96
Ala Leu Ser Ala Gln Ser Cys Gly Pro Gly Arg Gly Pro Val Gly Arg
             20                  25                  30

CGG CGT TAT GTG CGC AAG CAA CTT GTG CCT CTG CTA TAC AAG CAG TTT       144
Arg Arg Tyr Val Arg Lys Gln Leu Val Pro Leu Leu Tyr Lys Gln Phe
         35                  40                  45

GTG CCC AGT ATG CCC GAG CGG ACC CTG GGC GCG AGT GGG CCA GCG GAG       192
Val Pro Ser Met Pro Glu Arg Thr Leu Gly Ala Ser Gly Pro Ala Glu
     50                  55                  60

GGG AGG GTA ACA AGG GGG TCG GAG CGC TTC CGG GAC CTC GTA CCC AAC       240
Gly Arg Val Thr Arg Gly Ser Glu Arg Phe Arg Asp Leu Val Pro Asn
 65                  70                  75                  80

TAC AAC CCC GAC ATA ATC TTC AAG GAT GAG GAG AAC AGC GGC GCA GAC       288
Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Ser Gly Ala Asp
                 85                  90                  95

CGC CTG ATG ACA GAG CGT TGC AAA GAG CGG GTG AAC GCT CTA GCC ATC       336
Arg Leu Met Thr Glu Arg Cys Lys Glu Arg Val Asn Ala Leu Ala Ile
            100                 105                 110

GCG GTG ATG AAC ATG TGG CCC GGA GTA CGC CTA CGT GTG ACT GAA GGC       384
Ala Val Met Asn Met Trp Pro Gly Val Arg Leu Arg Val Thr Glu Gly
        115                 120                 125
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130 135 140

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Arg Ala Leu Asp Ile Thr Thr Ser Asp Arg Asp Arg Asn Lys Tyr Gly
145 150 155 160

TTG TTG GCG CGC CTA GCT GTG GAA GCC GGA TTC GAC TGG GTC TAC TAC 528
Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr
165 170 175

GAG TCC CGC AAC CAC ATC CAC GTA TCG GTC AAA GCT GAT AAC TCA CTG 576
Glu Ser Arg Asn His Ile His Val Ser Val Lys Ala Asp Asn Ser Leu
180 185 190

GCG GTC CGA GCC GGA GGC TGC TTT CCG GGA AAT GCC ACG GTG CGC TTG 624
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195 200 205

CGG AGC GGC GAA CGG AAG GGG CTG AGG GAA CTA CAT CGT GGT GAC TGG 672
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210 215 220

GTA CTG GCC GCT GAT GCA GCG GGC CGA GTG GTA CCC ACG CCA GTG CTG 720
Val Leu Ala Ala Asp Ala Ala Gly Arg Val Val Pro Thr Pro Val Leu
225 230 235 240

CTC TTC CTG GAC CGG GAT CTG CAG CGC CGC GCC TCG TTC GTG GCT GTG 768
Leu Phe Leu Asp Arg Asp Leu Gln Arg Arg Ala Ser Phe Val Ala Val
245 250 255

GAG ACC GAG CGG CCT CCG CGC AAA CTG TTG CTC ACA CCC TGG CAT CTG 816
Glu Thr Glu Arg Pro Pro Arg Lys Leu Leu Leu Thr Pro Trp His Leu
260 265 270

GTG TTC GCT GCT CGC GGG CCA GCG CCT GCT CCA GGT GAC TTT GCA CCG 864
Val Phe Ala Ala Arg Gly Pro Ala Pro Ala Pro Gly Asp Phe Ala Pro
275 280 285

GTG TTC GCG CGC CGC TTA CGT GCT GGC GAC TCG GTG CTG GCT CCC GGC 912
Val Phe Ala Arg Arg Leu Arg Ala Gly Asp Ser Val Leu Ala Pro Gly
290 295 300

GGG GAC GCG CTC CAG CCG GCG CGC GTA GCC CGC GTG GCG CGC GAG GAA 960
Gly Asp Ala Leu Gln Pro Ala Arg Val Ala Arg Val Ala Arg Glu Glu
305 310 315 320

GCC GTG GGC GTG TTC GCA CCG CTC ACT GCG CAC GGG ACG CTG CTG GTC 1008
Ala Val Gly Val Phe Ala Pro Leu Thr Ala His Gly Thr Leu Leu Val
325 330 335

AAC GAC GTC CTC GCC TCC TGC TAC GCG GTT CTA GAG AGT CAC CAG TGG 1056
Asn Asp Val Leu Ala Ser Cys Tyr Ala Val Leu Glu Ser His Gln Trp
340 345 350

GCC CAC CGC GCC TTC GCC CCT TTG CGG CTG CTG CAC GCG CTC GGG GCT 1104
Ala His Arg Ala Phe Ala Pro Leu Arg Leu Leu His Ala Leu Gly Ala
355 360 365

```
CTG CTC CCT GGG GGT GCA GTC CAG CCG ACT GGC ATG CAT TGG TAC TCT       1152
Leu Leu Pro Gly Gly Ala Val Gln Pro Thr Gly Met His Trp Tyr Ser
    370                 375                 380

CGC CTC CTT TAC CGC TTG GCC GAG GAG TTA ATG GCC TG                    1190
Arg Leu Leu Tyr Arg Leu Ala Glu Glu Leu Met Gly
385                 390                 395
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1281 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1233

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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ATG TCT CCC GCC TGG CTC CGG CCC CGA CTG CGG TTC TGT CTG TTC CTG         48
Met Ser Pro Ala Trp Leu Arg Pro Arg Leu Arg Phe Cys Leu Phe Leu
  1               5                  10                  15

CTG CTG CTG CTT CTG GTG CCG GCG GCG CGG GGC TGC GGG CCG GGC CGG         96
Leu Leu Leu Leu Leu Val Pro Ala Ala Arg Gly Cys Gly Pro Gly Arg
                 20                  25                  30

GTG GTG GGC AGC CGC CGG AGG CCG CCT CGC AAG CTC GTG CCT CTT GCC        144
Val Val Gly Ser Arg Arg Arg Pro Pro Arg Lys Leu Val Pro Leu Ala
         35                  40                  45

TAC AAG CAG TTC AGC CCC AAC GTG CCG GAG AAG ACC CTG GGC GCC AGC        192
Tyr Lys Gln Phe Ser Pro Asn Val Pro Glu Lys Thr Leu Gly Ala Ser
     50                  55                  60

GGG CGC TAC GAA GGC AAG ATC GCG CGC AGC TCT GAG CGC TTC AAA GAG        240
Gly Arg Tyr Glu Gly Lys Ile Ala Arg Ser Ser Glu Arg Phe Lys Glu
 65                  70                  75                  80

CTC ACC CCC AAC TAC AAT CCC GAC ATC ATC TTC AAG GAC GAG GAG AAC        288
Leu Thr Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn
                 85                  90                  95

ACG GGT GCC GAC CGC CTC ATG ACC CAG CGC TGC AAG GAC CGT CTG AAC        336
Thr Gly Ala Asp Arg Leu Met Thr Gln Arg Cys Lys Asp Arg Leu Asn
            100                 105                 110

TCA CTG GCC ATC TCT GTC ATG AAC CAG TGG CCT GGT GTG AAA CTG CGG        384
Ser Leu Ala Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg
        115                 120                 125
```

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GTG ACC GAA GGC CGG GAT GAA GAT GGC CAT CAC TCA GAG GAG TCT TTA      432
Val Thr Glu Gly Arg Asp Glu Asp Gly His His Ser Glu Glu Ser Leu
    130                 135                 140

CAC TAT GAG GGC CGC GCG GTG GAT ATC ACC ACC TCA GAC CGT GAC CGA      480
His Tyr Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg
145                 150                 155                 160

AAT AAG TAT GGA CTG CTG GCG CGC TTA GCA GTG GAG GCC GGC TTC GAC      528
Asn Lys Tyr Gly Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp
                165                 170                 175

TGG GTG TAT TAC GAG TCC AAG GCC CAC GTG CAT TGC TCT GTC AAG TCT      576
Trp Val Tyr Tyr Glu Ser Lys Ala His Val His Cys Ser Val Lys Ser
            180                 185                 190

GAG CAT TCG GCC GCT GCC AAG ACA GGT GGC TGC TTT CCT GCC GGA GCC      624
Glu His Ser Ala Ala Ala Lys Thr Gly Gly Cys Phe Pro Ala Gly Ala
        195                 200                 205

CAG GTG CGC CTA GAG AAC GGG GAG CGT GTG GCC CTG TCA GCT GTA AAG      672
Gln Val Arg Leu Glu Asn Gly Glu Arg Val Ala Leu Ser Ala Val Lys
    210                 215                 220

CCA GGA GAC CGG GTG CTG GCC ATG GGG GAG GAT GGG ACC CCC ACC TTC      720
Pro Gly Asp Arg Val Leu Ala Met Gly Glu Asp Gly Thr Pro Thr Phe
225                 230                 235                 240

AGT GAT GTG CTT ATT TTC CTG GAC CGC GAG CCA AAC CGG CTG AGA GCT      768
Ser Asp Val Leu Ile Phe Leu Asp Arg Glu Pro Asn Arg Leu Arg Ala
                245                 250                 255

TTC CAG GTC ATC GAG ACT CAG GAT CCT CCG CGT CGG CTG GCG CTC ACG      816
Phe Gln Val Ile Glu Thr Gln Asp Pro Pro Arg Arg Leu Ala Leu Thr
            260                 265                 270

CCT GCC CAC CTG CTC TTC ATT GCG GAC AAT CAT ACA GAA CCA GCA GCC      864
Pro Ala His Leu Leu Phe Ile Ala Asp Asn His Thr Glu Pro Ala Ala
        275                 280                 285

CAC TTC CGG GCC ACA TTT GCC AGC CAT GTG CAA CCA GGC CAA TAT GTG      912
His Phe Arg Ala Thr Phe Ala Ser His Val Gln Pro Gly Gln Tyr Val
    290                 295                 300

CTG GTA TCA GGG GTA CCA GGC CTC CAG CCT GCT CGG GTG GCA GCT GTC      960
Leu Val Ser Gly Val Pro Gly Leu Gln Pro Ala Arg Val Ala Ala Val
305                 310                 315                 320

TCC ACC CAC GTG GCC CTT GGG TCC TAT GCT CCT CTC ACA AGG CAT GGG     1008
Ser Thr His Val Ala Leu Gly Ser Tyr Ala Pro Leu Thr Arg His Gly
                325                 330                 335

ACA CTT GTG GTG GAG GAT GTG GTG GCC TCC TGC TTT GCA GCT GTG GCT     1056
Thr Leu Val Val Glu Asp Val Val Ala Ser Cys Phe Ala Ala Val Ala
            340                 345                 350

GAC CAC CAT CTG GCT CAG TTG GCC TTC TGG CCC CTG CGA CTG TTT CCC     1104
Asp His His Leu Ala Gln Leu Ala Phe Trp Pro Leu Arg Leu Phe Pro
        355                 360                 365
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AGT TTG GCA TGG GGC AGC TGG ACC CCA AGT GAG GGT GTT CAC TCC TAC      1152
Ser Leu Ala Trp Gly Ser Trp Thr Pro Ser Glu Gly Val His Ser Tyr
    370                 375                 380

CCT CAG ATG CTC TAC CGC CTG GGG CGT CTC TTG CTA GAA GAG AGC ACC      1200
Pro Gln Met Leu Tyr Arg Leu Gly Arg Leu Leu Leu Glu Glu Ser Thr
385                 390                 395                 400

TTC CAT CCA CTG GGC ATG TCT GGG GCA GGA AGC TGAAGGGACT CTAACCACTG    1253
Phe His Pro Leu Gly Met Ser Gly Ala Gly Ser
                405                 410

CCCTCCTGGA ACTGCTGTGC GTGGATCC                                       1281
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1313 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1314

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
ATG CTG CTG CTG CTG GCC AGA TGT TTT CTG GTG ATC CTT GCT TCC TCG        48
Met Leu Leu Leu Leu Ala Arg Cys Phe Leu Val Ile Leu Ala Ser Ser
  1               5                  10                  15

CTG CTG GTG TGC CCC GGG CTG GCC TGT GGG CCC GGC AGG GGG TTT GGA        96
Leu Leu Val Cys Pro Gly Leu Ala Cys Gly Pro Gly Arg Gly Phe Gly
            20                  25                  30

AAG AGG CGG CAC CCC AAA AAG CTG ACC CCT TTA GCC TAC AAG CAG TTT       144
Lys Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe
        35                  40                  45

ATT CCC AAC GTA GCC GAG AAG ACC CTA GGG GCC AGC GGC AGA TAT GAA       192
Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu
    50                  55                  60

GGG AAG ATC ACA AGA AAC TCC GAA CGA TTT AAG GAA CTC ACC CCC AAT       240
Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn
65                  70                  75                  80

TAC AAC CCC GAC ATC ATA TTT AAG GAT GAG GAA AAC ACG GGA GCA GAC       288
Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp
                85                  90                  95

CGG CTG ATG ACT CAG AGG TGC AAA GAC AAG TTA AAT GCC TTG GCC ATC       336
Arg Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu Ala Ile
            100                 105                 110
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TCT GTG ATG AAC CAG TGG CCT GGA GTG AGG CTG CGA GTG ACC GAG GGC        384
Ser Val Met Asn Gln Trp Pro Gly Val Arg Leu Arg Val Thr Glu Gly
        115                 120                 125

TGG GAT GAG GAC GGC CAT CAT TCA GAG GAG TCT CTA CAC TAT GAG GGT        432
Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr Glu Gly
    130                 135                 140

CGA GCA GTG GAC ATC ACC ACG TCC GAC CGG GAC CGC AGC AAG TAC GGC        480
Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Ser Lys Tyr Gly
145                 150                 155                 160

ATG CTG GCT CGC CTG GCT GTG GAA GCA GGT TTC GAC TGG GTC TAC TAT        528
Met Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr
                165                 170                 175

GAA TCC AAA GCT CAC ATC CAC TGT TCT GTG AAA GCA GAG AAC TCC GTG        576
Glu Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn Ser Val
            180                 185                 190

GCG GCC AAA TCC GGC GGC TGT TTC CCG GGA TCC GCC ACC GTG CAC CTG        624
Ala Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Thr Val His Leu
        195                 200                 205

GAG CAG GGC GGC ACC AAG CTG GTG AAG GAC TTA CGT CCC GGA GAC CGC        672
Glu Gln Gly Gly Thr Lys Leu Val Lys Asp Leu Arg Pro Gly Asp Arg
    210                 215                 220

GTG CTG GCG GCT GAC GAC CAG GGC CGG CTG CTG TAC AGC GAC TTC CTC        720
Val Leu Ala Ala Asp Asp Gln Gly Arg Leu Leu Tyr Ser Asp Phe Leu
225                 230                 235                 240

ACC TTC CTG GAC CGC GAC GAA GGC GCC AAG AAG GTC TTC TAC GTG ATC        768
Thr Phe Leu Asp Arg Asp Glu Gly Ala Lys Lys Val Phe Tyr Val Ile
                245                 250                 255

GAG ACG CTG GAG CCG CGC GAG CGC CTG CTG CTC ACC GCC GCG CAC CTG        816
Glu Thr Leu Glu Pro Arg Glu Arg Leu Leu Leu Thr Ala Ala His Leu
            260                 265                 270

CTC TTC GTG GCG CCG CAC AAC GAC TCG GGG CCC ACG CCC GGG CCA AGC        864
Leu Phe Val Ala Pro His Asn Asp Ser Gly Pro Thr Pro Gly Pro Ser
        275                 280                 285

GCG CTC TTT GCC AGC CGC GTG CGC CCC GGG CAG CGC GTG TAC GTG GTG        912
Ala Leu Phe Ala Ser Arg Val Arg Pro Gly Gln Arg Val Tyr Val Val
    290                 295                 300

GCT GAA CGC GGC GGG GAC CGC CGG CTG CTG CCC GCC GCG GTG CAC AGC        960
Ala Glu Arg Gly Gly Asp Arg Arg Leu Leu Pro Ala Ala Val His Ser
305                 310                 315                 320

GTG ACG CTG CGA GAG GAG GAG GCG GGC GCG TAC GCG CCG CTC ACG GCG       1008
Val Thr Leu Arg Glu Glu Glu Ala Gly Ala Tyr Ala Pro Leu Thr Ala
                325                 330                 335

CAC GGC ACC ATT CTC ATC AAC CGG GTG CTC GCC TCG TGC TAC GCT GTC       1056
His Gly Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys Tyr Ala Val
            340                 345                 350
```

ATC GAG GAG CAC AGC TGG GCA CAC CGG GCC TTC GCG CCT TTC CGC CTG 1104
Ile Glu Glu His Ser Trp Ala His Arg Ala Phe Ala Pro Phe Arg Leu
355 360 365

GCG CAC GCG CTG CTG GCC GCG CTG GCA CCC GCC CGC ACG GAC GGC GGG 1152
Ala His Ala Leu Leu Ala Ala Leu Ala Pro Ala Arg Thr Asp Gly Gly
370 375 380

GGC GGG GGC AGC ATC CCT GCA GCG CAA TCT GCA ACG GAA GCG AGG GGC 1200
Gly Gly Gly Ser Ile Pro Ala Ala Gln Ser Ala Thr Glu Ala Arg Gly
385 390 395 400

GCG GAG CCG ACT GCG GGC ATC CAC TGG TAC TCG CAG CTG CTC TAC CAC 1248
Ala Glu Pro Thr Ala Gly Ile His Trp Tyr Ser Gln Leu Leu Tyr His
405 410 415

ATT GGC ACC TGG CTG TTG GAC AGC GAG ACC ATG CAT CCC TTG GGA ATG 1296
Ile Gly Thr Trp Leu Leu Asp Ser Glu Thr Met His Pro Leu Gly Met
420 425 430

GCG GTC AAG TCC AGC TG 1313
Ala Val Lys Ser Ser
435

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1256 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1257

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATG CGG CTT TTG ACG AGA GTG CTG CTG GTG TCT CTT CTC ACT CTG TCC 48
Met Arg Leu Leu Thr Arg Val Leu Leu Val Ser Leu Leu Thr Leu Ser
1 5 10 15

TTG GTG GTG TCC GGA CTG GCC TGC GGT CCT GGC AGA GGC TAC GGC AGA 96
Leu Val Val Ser Gly Leu Ala Cys Gly Pro Gly Arg Gly Tyr Gly Arg
20 25 30

AGA AGA CAT CCG AAG AAG CTG ACA CCT CTC GCC TAC AAG CAG TTC ATA 144
Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe Ile
35 40 45

CCT AAT GTC GCG GAG AAG ACC TTA GGG GCC AGC GGC AGA TAC GAG GGC 192
Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu Gly
50 55 60

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AAG ATA ACG CGC AAT TCG GAG AGA TTT AAA GAA CTT ACT CCA AAT TAC      240
Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn Tyr
 65                  70                  75                  80

AAT CCC GAC ATT ATC TTT AAG GAT GAG GAG AAC ACG GGA GCG GAC AGG      288
Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp Arg
                 85                  90                  95

CTC ATG ACA CAG AGA TGC AAA GAC AAG CTG AAC TCG CTG GCC ATC TCT      336
Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ser Leu Ala Ile Ser
            100                 105                 110

GTA ATG AAC CAC TGG CCA GGG GTT AAG CTG CGT GTG ACA GAG GGC TGG      384
Val Met Asn His Trp Pro Gly Val Lys Leu Arg Val Thr Glu Gly Trp
        115                 120                 125

GAT GAG GAC GGT CAC CAT TTT GAA GAA TCA CTC CAC TAC GAG GGA AGA      432
Asp Glu Asp Gly His His Phe Glu Glu Ser Leu His Tyr Glu Gly Arg
    130                 135                 140

GCT GTT GAT ATT ACC ACC TCT GAC CGA GAC AAG AGC AAA TAC GGG ACA      480
Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Lys Ser Lys Tyr Gly Thr
145                 150                 155                 160

CTG TCT CGC CTA GCT GTG GAG GCT GGA TTT GAC TGG GTC TAT TAC GAG      528
Leu Ser Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr Glu
                165                 170                 175

TCC AAA GCC CAC ATT CAT TGC TCT GTC AAA GCA GAA AAT TCG GTT GCT      576
Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn Ser Val Ala
            180                 185                 190

GCG AAA TCT GGG GGC TGT TTC CCA GGT TCG GCT CTG GTC TCG CTC CAG      624
Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Leu Val Ser Leu Gln
        195                 200                 205

GAC GGA GGA CAG AAG GCC GTG AAG GAC CTG AAC CCC GGA GAC AAG GTG      672
Asp Gly Gly Gln Lys Ala Val Lys Asp Leu Asn Pro Gly Asp Lys Val
    210                 215                 220

CTG GCG GCA GAC AGC GCG GGA AAC CTG GTG TTC AGC GAC TTC ATC ATG      720
Leu Ala Ala Asp Ser Ala Gly Asn Leu Val Phe Ser Asp Phe Ile Met
225                 230                 235                 240

TTC ACA GAC CGA GAC TCC ACG ACG CGA CGT GTG TTT TAC GTC ATA GAA      768
Phe Thr Asp Arg Asp Ser Thr Thr Arg Arg Val Phe Tyr Val Ile Glu
                245                 250                 255

ACG CAA GAA CCC GTT GAA AAG ATC ACC CTC ACC GCC GCT CAC CTC CTT      816
Thr Gln Glu Pro Val Glu Lys Ile Thr Leu Thr Ala Ala His Leu Leu
            260                 265                 270

TTT GTC CTC GAC AAC TCA ACG GAA GAT CTC CAC ACC ATG ACC GCC GCG      864
Phe Val Leu Asp Asn Ser Thr Glu Asp Leu His Thr Met Thr Ala Ala
        275                 280                 285

TAT GCC AGC AGT GTC AGA GCC GGA CAA AAG GTG ATG GTT GTT GAT GAT      912
Tyr Ala Ser Ser Val Arg Ala Gly Gln Lys Val Met Val Val Asp Asp
    290                 295                 300
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AGC GGT CAG CTT AAA TCT GTC ATC GTG CAG CGG ATA TAC ACG GAG GAG 960
Ser Gly Gln Leu Lys Ser Val Ile Val Gln Arg Ile Tyr Thr Glu Glu
305 310 315 320

CAG CGG GGC TCG TTC GCA CCA GTG ACT GCA CAT GGG ACC ATT GTG GTC 1008
Gln Arg Gly Ser Phe Ala Pro Val Thr Ala His Gly Thr Ile Val Val
325 330 335

GAC AGA ATA CTG GCG TCC TGT TAC GCC GTA ATA GAG GAC CAG GGG CTT 1056
Asp Arg Ile Leu Ala Ser Cys Tyr Ala Val Ile Glu Asp Gln Gly Leu
340 345 350

GCG CAT TTG GCC TTC GCG CCC GCC AGG CTC TAT TAT TAC GTG TCA TCA 1104
Ala His Leu Ala Phe Ala Pro Ala Arg Leu Tyr Tyr Tyr Val Ser Ser
355 360 365

TTC CTG TCC CCC AAA ACT CCA GCA GTC GGT CCA ATG CGA CTT TAC AAC 1152
Phe Leu Ser Pro Lys Thr Pro Ala Val Gly Pro Met Arg Leu Tyr Asn
370 375 380

AGG AGG GGG TCC ACT GGT ACT CCA GGC TCC TGT CAT CAA ATG GGA ACG 1200
Arg Arg Gly Ser Thr Gly Thr Pro Gly Ser Cys His Gln Met Gly Thr
385 390 395 400

TGG CTT TTG GAC AGC AAC ATG CTT CAT CCT TTG GGG ATG TCA GTA AAC 1248
Trp Leu Leu Asp Ser Asn Met Leu His Pro Leu Gly Met Ser Val Asn
405 410 415

TCA AGC TG 1256
Ser Ser

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1425 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1425

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATG CTG CTG CTG GCG AGA TGT CTG CTG CTA GTC CTC GTC TCC TCG CTG 48
Met Leu Leu Leu Ala Arg Cys Leu Leu Leu Val Leu Val Ser Ser Leu
1 5 10 15

CTG GTA TGC TCG GGA CTG GCG TGC GGA CCG GGC AGG GGG TTC GGG AAG 96
Leu Val Cys Ser Gly Leu Ala Cys Gly Pro Gly Arg Gly Phe Gly Lys
20 25 30

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AGG AGG CAC CCC AAA AAG CTG ACC CCT TTA GCC TAC AAG CAG TTT ATC        144
Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe Ile
         35                      40                      45

CCC AAT GTG GCC GAG AAG ACC CTA GGC GCC AGC GGA AGG TAT GAA GGG        192
Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu Gly
     50                      55                      60

AAG ATC TCC AGA AAC TCC GAG CGA TTT AAG GAA CTC ACC CCC AAT TAC        240
Lys Ile Ser Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn Tyr
 65                      70                      75                  80

AAC CCC GAC ATC ATA TTT AAG GAT GAA GAA AAC ACC GGA GCG GAC AGG        288
Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp Arg
                 85                      90                      95

CTG ATG ACT CAG AGG TGT AAG GAC AAG TTG AAC GCT TTG GCC ATC TCG        336
Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu Ala Ile Ser
            100                     105                     110

GTG ATG AAC CAG TGG CCA GGA GTG AAA CTG CGG GTG ACC GAG GGC TGG        384
Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg Val Thr Glu Gly Trp
        115                     120                     125

GAC GAA GAT GGC CAC CAC TCA GAG GAG TCT CTG CAC TAC GAG GGC CGC        432
Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr Glu Gly Arg
    130                     135                     140

GCA GTG GAC ATC ACC ACG TCT GAC CGC GAC CGC AGC AAG TAC GGC ATG        480
Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Ser Lys Tyr Gly Met
145                     150                     155                     160

CTG GCC CGC CTG GCG GTG GAG GCC GGC TTC GAC TGG GTG TAC TAC GAG        528
Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr Glu
                165                     170                     175

TCC AAG GCA CAT ATC CAC TGC TCG GTG AAA GCA GAG AAC TCG GTG GCG        576
Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn Ser Val Ala
            180                     185                     190

GCC AAA TCG GGA GGC TGC TTC CCG GGC TCG GCC ACG GTG CAC CTG GAG        624
Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Thr Val His Leu Glu
        195                     200                     205

CAG GGC GGC ACC AAG CTG GTG AAG GAC CTG AGC CCC GGG GAC CGC GTG        672
Gln Gly Gly Thr Lys Leu Val Lys Asp Leu Ser Pro Gly Asp Arg Val
    210                     215                     220

CTG GCG GCG GAC GAC CAG GGC CGG CTG CTC TAC AGC GAC TTC CTC ACT        720
Leu Ala Ala Asp Asp Gln Gly Arg Leu Leu Tyr Ser Asp Phe Leu Thr
225                     230                     235                     240

TTC CTG GAC CGC GAC GAC GGC GCC AAG AAG GTC TTC TAC GTG ATC GAG        768
Phe Leu Asp Arg Asp Asp Gly Ala Lys Lys Val Phe Tyr Val Ile Glu
                245                     250                     255

ACG CGG GAG CCG CGC GAG CGC CTG CTG CTC ACC GCC GCG CAC CTG CTC        816
Thr Arg Glu Pro Arg Glu Arg Leu Leu Leu Thr Ala Ala His Leu Leu
            260                     265                     270
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TTT GTG GCG CCG CAC AAC GAC TCG GCC ACC GGG GAG CCC GAG GCG TCC        864
Phe Val Ala Pro His Asn Asp Ser Ala Thr Gly Glu Pro Glu Ala Ser
        275                 280                 285

TCG GGC TCG GGG CCG CCT TCC GGG GGC GCA CTG GGG CCT CGG GCG CTG        912
Ser Gly Ser Gly Pro Pro Ser Gly Gly Ala Leu Gly Pro Arg Ala Leu
    290                 295                 300

TTC GCC AGC CGC GTG CGC CCG GGC CAG CGC GTG TAC GTG GTG GCC GAG        960
Phe Ala Ser Arg Val Arg Pro Gly Gln Arg Val Tyr Val Val Ala Glu
305                 310                 315                 320

CGT GAC GGG GAC CGC CGG CTC CTG CCC GCC GCT GTG CAC AGC GTG ACC       1008
Arg Asp Gly Asp Arg Arg Leu Leu Pro Ala Ala Val His Ser Val Thr
                325                 330                 335

CTA AGC GAG GAG GCC GCG GGC GCC TAC GCG CCG CTC ACG GCC CAG GGC       1056
Leu Ser Glu Glu Ala Ala Gly Ala Tyr Ala Pro Leu Thr Ala Gln Gly
            340                 345                 350

ACC ATT CTC ATC AAC CGG GTG CTG GCC TCG TGC TAC GCG GTC ATC GAG       1104
Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys Tyr Ala Val Ile Glu
        355                 360                 365

GAG CAC AGC TGG GCG CAC CGG GCC TTC GCG CCC TTC CGC CTG GCG CAC       1152
Glu His Ser Trp Ala His Arg Ala Phe Ala Pro Phe Arg Leu Ala His
    370                 375                 380

GCG CTC CTG GCT GCA CTG GCG CCC GCG CGC ACG GAC CGC GGC GGG GAC       1200
Ala Leu Leu Ala Ala Leu Ala Pro Ala Arg Thr Asp Arg Gly Gly Asp
385                 390                 395                 400

AGC GGC GGC GGG GAC CGC GGG GGC GGC GGC GGC AGA GTA GCC CTA ACC       1248
Ser Gly Gly Gly Asp Arg Gly Gly Gly Gly Gly Arg Val Ala Leu Thr
                405                 410                 415

GCT CCA GGT GCT GCC GAC GCT CCG GGT GCG GGG GCC ACC GCG GGC ATC       1296
Ala Pro Gly Ala Ala Asp Ala Pro Gly Ala Gly Ala Thr Ala Gly Ile
            420                 425                 430

CAC TGG TAC TCG CAG CTG CTC TAC CAA ATA GGC ACC TGG CTC CTG GAC       1344
His Trp Tyr Ser Gln Leu Leu Tyr Gln Ile Gly Thr Trp Leu Leu Asp
        435                 440                 445

AGC GAG GCC CTG CAC CCG CTG GGC ATG GCG GTC AAG TCC AGC NNN AGC       1392
Ser Glu Ala Leu His Pro Leu Gly Met Ala Val Lys Ser Ser Xaa Ser
    450                 455                 460

CGG GGG GCC GGG GGA GGG GCG CGG GAG GGG GCC                           1425
Arg Gly Ala Gly Gly Gly Ala Arg Glu Gly Ala
465                 470                 475
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1622 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 51..1283

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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CATCAGCCCA CCAGGAGACC TCGCCCGCCG CTCCCCCGGG CTCCCCGGCC ATG TCT         56
                                                      Met Ser
                                                        1

CCC GCC CGG CTC CGG CCC CGA CTG CAC TTC TGC CTG GTC CTG TTG CTG       104
Pro Ala Arg Leu Arg Pro Arg Leu His Phe Cys Leu Val Leu Leu Leu
          5                  10                  15

CTG CTG GTG GTG CCC GCG GCA TGG GGC TGC GGG CCG GGT CGG GTG GTG       152
Leu Leu Val Val Pro Ala Ala Trp Gly Cys Gly Pro Gly Arg Val Val
     20                  25                  30

GGC AGC CGC CGG CGA CCG CCA CGC AAA CTC GTG CCG CTC GCC TAC AAG       200
Gly Ser Arg Arg Arg Pro Pro Arg Lys Leu Val Pro Leu Ala Tyr Lys
 35                  40                  45                  50

CAG TTC AGC CCC AAT GTG CCC GAG AAG ACC CTG GGC GCC AGC GGA CGC       248
Gln Phe Ser Pro Asn Val Pro Glu Lys Thr Leu Gly Ala Ser Gly Arg
                 55                  60                  65

TAT GAA GGC AAG ATC GCT CGC AGC TCC GAG CGC TTC AAG GAG CTC ACC       296
Tyr Glu Gly Lys Ile Ala Arg Ser Ser Glu Arg Phe Lys Glu Leu Thr
             70                  75                  80

CCC AAT TAC AAT CCA GAC ATC ATC TTC AAG GAC GAG GAG AAC ACA GGC       344
Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly
         85                  90                  95

GCC GAC CGC CTC ATG ACC CAG CGC TGC AAG GAC CGC CTG AAC TCG CTG       392
Ala Asp Arg Leu Met Thr Gln Arg Cys Lys Asp Arg Leu Asn Ser Leu
    100                 105                 110

GCT ATC TCG GTG ATG AAC CAG TGG CCC GGT GTG AAG CTG CGG GTG ACC       440
Ala Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg Val Thr
115                 120                 125                 130

GAG GGC TGG GAC GAG GAC GGC CAC CAC TCA GAG GAG TCC CTG CAT TAT       488
Glu Gly Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr
                135                 140                 145

GAG GGC CGC GCG GTG GAC ATC ACC ACA TCA GAC CGC GAC CGC AAT AAG       536
Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Asn Lys
            150                 155                 160

TAT GGA CTG CTG GCG CGC TTG GCA GTG GAG GCC GGC TTT GAC TGG GTG       584
Tyr Gly Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val
        165                 170                 175
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TAT TAC GAG TCA AAG GCC CAC GTG CAT TGC TCC GTC AAG TCC GAG CAC      632
Tyr Tyr Glu Ser Lys Ala His Val His Cys Ser Val Lys Ser Glu His
    180                 185                 190

TCG GCC GCA GCC AAG ACG GGC GGC TGC TTC CCT GCC GGA GCC CAG GTA      680
Ser Ala Ala Ala Lys Thr Gly Gly Cys Phe Pro Ala Gly Ala Gln Val
195                 200                 205                 210

CGC CTG GAG AGT GGG GCG CGT GTG GCC TTG TCA GCC GTG AGG CCG GGA      728
Arg Leu Glu Ser Gly Ala Arg Val Ala Leu Ser Ala Val Arg Pro Gly
                215                 220                 225

GAC CGT GTG CTG GCC ATG GGG GAG GAT GGG AGC CCC ACC TTC AGC GAT      776
Asp Arg Val Leu Ala Met Gly Glu Asp Gly Ser Pro Thr Phe Ser Asp
            230                 235                 240

GTG CTC ATT TTC CTG GAC CGC GAG CCC CAC AGG CTG AGA GCC TTC CAG      824
Val Leu Ile Phe Leu Asp Arg Glu Pro His Arg Leu Arg Ala Phe Gln
        245                 250                 255

GTC ATC GAG ACT CAG GAC CCC CCA CGC CGC CTG GCA CTC ACA CCC GCT      872
Val Ile Glu Thr Gln Asp Pro Pro Arg Arg Leu Ala Leu Thr Pro Ala
    260                 265                 270

CAC CTG CTC TTT ACG GCT GAC AAT CAC ACG GAG CCG GCA GCC CGC TTC      920
His Leu Leu Phe Thr Ala Asp Asn His Thr Glu Pro Ala Ala Arg Phe
275                 280                 285                 290

CGG GCC ACA TTT GCC AGC CAC GTG CAG CCT GGC CAG TAC GTG CTG GTG      968
Arg Ala Thr Phe Ala Ser His Val Gln Pro Gly Gln Tyr Val Leu Val
                295                 300                 305

GCT GGG GTG CCA GGC CTG CAG CCT GCC CGC GTG GCA GCT GTC TCT ACA     1016
Ala Gly Val Pro Gly Leu Gln Pro Ala Arg Val Ala Ala Val Ser Thr
            310                 315                 320

CAC GTG GCC CTC GGG GCC TAC GCC CCG CTC ACA AAG CAT GGG ACA CTG     1064
His Val Ala Leu Gly Ala Tyr Ala Pro Leu Thr Lys His Gly Thr Leu
        325                 330                 335

GTG GTG GAG GAT GTG GTG GCA TCC TGC TTC GCG GCC GTG GCT GAC CAC     1112
Val Val Glu Asp Val Val Ala Ser Cys Phe Ala Ala Val Ala Asp His
    340                 345                 350

CAC CTG GCT CAG TTG GCC TTC TGG CCC CTG AGA CTC TTT CAC AGC TTG     1160
His Leu Ala Gln Leu Ala Phe Trp Pro Leu Arg Leu Phe His Ser Leu
355                 360                 365                 370

GCA TGG GGC AGC TGG ACC CCG GGG GAG GGT GTG CAT TGG TAC CCC CAG     1208
Ala Trp Gly Ser Trp Thr Pro Gly Glu Gly Val His Trp Tyr Pro Gln
                375                 380                 385

CTG CTC TAC CGC CTG GGG CGT CTC CTG CTA GAA GAG GGC AGC TTC CAC     1256
Leu Leu Tyr Arg Leu Gly Arg Leu Leu Leu Glu Glu Gly Ser Phe His
            390                 395                 400

CCA CTG GGC ATG TCC GGG GCA GGG AGC TGAAAGGACT CCACCGCTGC           1303
Pro Leu Gly Met Ser Gly Ala Gly Ser
        405                 410
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CCTCCTGGAA CTGCTGTACT GGGTCCAGAA GCCTCTCAGC CAGGAGGGAG CTGGCCCTGG      1363

AAGGGACCTG AGCTGGGGGA CACTGGCTCC TGCCATCTCC TCTGCCATGA AGATACACCA      1423

TTGAGACTTG ACTGGGCAAC ACCAGCGTCC CCCACCCGCG TCGTGGTGTA GTCATAGAGC      1483

TGCAAGCTGA GCTGGCGAGG GGATGGTTGT TGACCCCTCT CTCCTAGAGA CCTTGAGGCT      1543

GGCACGGCGA CTCCCAACTC AGCCTGCTCT CACTACGAGT TTTCATACTC TGCCTCCCCC      1603

ATTGGGAGGG CCCATTCCC                                                   1622
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1191 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1191

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
ATG GCT CTC CTG ACC AAT CTA CTG CCC TTG TGC TGC TTG GCA CTT CTG        48
Met Ala Leu Leu Thr Asn Leu Leu Pro Leu Cys Cys Leu Ala Leu Leu
  1               5                  10                  15

GCG CTG CCA GCC CAG AGC TGC GGG CCG GGC CGG GGG CCG GTT GGC CGG        96
Ala Leu Pro Ala Gln Ser Cys Gly Pro Gly Arg Gly Pro Val Gly Arg
             20                  25                  30

CGC CGC TAT GCG CGC AAG CAG CTC GTG CCG CTA CTC TAC AAG CAA TTT       144
Arg Arg Tyr Ala Arg Lys Gln Leu Val Pro Leu Leu Tyr Lys Gln Phe
         35                  40                  45

GTG CCC GGC GTG CCA GAG CGG ACC CTG GGC GCC AGT GGG CCA GCG GAG       192
Val Pro Gly Val Pro Glu Arg Thr Leu Gly Ala Ser Gly Pro Ala Glu
     50                  55                  60

GGG AGG GTG GCA AGG GGC TCC GAG CGC TTC CGG GAC CTC GTG CCC AAC       240
Gly Arg Val Ala Arg Gly Ser Glu Arg Phe Arg Asp Leu Val Pro Asn
 65                  70                  75                  80

TAC AAC CCC GAC ATC ATC TTC AAG GAT GAG GAG AAC AGT GGA GCC GAC       288
Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Ser Gly Ala Asp
                 85                  90                  95

CGC CTG ATG ACC GAG CGT TGC AAG GAG AGG GTG AAC GCT TTG GCC ATT       336
Arg Leu Met Thr Glu Arg Cys Lys Glu Arg Val Asn Ala Leu Ala Ile
            100                 105                 110

GCC GTG ATG AAC ATG TGG CCC GGA GTG CGC CTA CGA GTG ACT GAG GGC       384
Ala Val Met Asn Met Trp Pro Gly Val Arg Leu Arg Val Thr Glu Gly
        115                 120                 125
```

TGG GAC GAG GAC GGC CAC CAC GCT CAG GAT TCA CTC CAC TAC GAA GGC 432
Trp Asp Glu Asp Gly His His Ala Gln Asp Ser Leu His Tyr Glu Gly
130 135 140

CGT GCT TTG GAC ATC ACT ACG TCT GAC CGC GAC CGC AAC AAG TAT GGG 480
Arg Ala Leu Asp Ile Thr Thr Ser Asp Arg Asp Arg Asn Lys Tyr Gly
145 150 155 160

TTG CTG GCG CGC CTC GCA GTG GAA GCC GGC TTC GAC TGG GTC TAC TAC 528
Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr
165 170 175

GAG TCC CGC AAC CAC GTC CAC GTG TCG GTC AAA GCT GAT AAC TCA CTG 576
Glu Ser Arg Asn His Val His Val Ser Val Lys Ala Asp Asn Ser Leu
180 185 190

GCG GTC CGG GCG GGC GGC TGC TTT CCG GGA AAT GCA ACT GTG CGC CTG 624
Ala Val Arg Ala Gly Gly Cys Phe Pro Gly Asn Ala Thr Val Arg Leu
195 200 205

TGG AGC GGC GAG CGG AAA GGG CTG CGG GAA CTG CAC CGC GGA GAC TGG 672
Trp Ser Gly Glu Arg Lys Gly Leu Arg Glu Leu His Arg Gly Asp Trp
210 215 220

GTT TTG GCG GCC GAT GCG TCA GGC CGG GTG GTG CCC ACG CCG GTG CTG 720
Val Leu Ala Ala Asp Ala Ser Gly Arg Val Val Pro Thr Pro Val Leu
225 230 235 240

CTC TTC CTG GAC CGG GAC TTG CAG CGC CGG GCT TCA TTT GTG GCT GTG 768
Leu Phe Leu Asp Arg Asp Leu Gln Arg Arg Ala Ser Phe Val Ala Val
245 250 255

GAG ACC GAG TGG CCT CCA CGC AAA CTG TTG CTC ACG CCC TGG CAC CTG 816
Glu Thr Glu Trp Pro Pro Arg Lys Leu Leu Leu Thr Pro Trp His Leu
260 265 270

GTG TTT GCC GCT CGA GGG CCG GCG CCC GCG CCA GGC GAC TTT GCA CCG 864
Val Phe Ala Ala Arg Gly Pro Ala Pro Ala Pro Gly Asp Phe Ala Pro
275 280 285

GTG TTC GCG CGC CGG CTA CGC GCT GGG GAC TCG GTG CTG GCG CCC GGC 912
Val Phe Ala Arg Arg Leu Arg Ala Gly Asp Ser Val Leu Ala Pro Gly
290 295 300

GGG GAT GCG CTT CGG CCA GCG CGC GTG GCC CGT GTG GCG CGG GAG GAA 960
Gly Asp Ala Leu Arg Pro Ala Arg Val Ala Arg Val Ala Arg Glu Glu
305 310 315 320

GCC GTG GGC GTG TTC GCG CCG CTC ACC GCG CAC GGG ACG CTG CTG GTG 1008
Ala Val Gly Val Phe Ala Pro Leu Thr Ala His Gly Thr Leu Leu Val
325 330 335

AAC GAT GTC CTG GCC TCT TGC TAC GCG GTT CTG GAG AGT CAC CAG TGG 1056
Asn Asp Val Leu Ala Ser Cys Tyr Ala Val Leu Glu Ser His Gln Trp
340 345 350

GCG CAC CGC GCT TTT GCC CCC TTG AGA CTG CTG CAC GCG CTA GGG GCG 1104
Ala His Arg Ala Phe Ala Pro Leu Arg Leu Leu His Ala Leu Gly Ala
355 360 365

```
CTG CTC CCC GGC GGG GCC GTC CAG CCG ACT GGC ATG CAT TGG TAC TCT      1152
Leu Leu Pro Gly Gly Ala Val Gln Pro Thr Gly Met His Trp Tyr Ser
    370                 375                 380

CGG CTC CTC TAC CGC TTA GCG GAG GAG CTA CTG GGC TG                   1191
Arg Leu Leu Tyr Arg Leu Ala Glu Glu Leu Leu Gly
385                 390                 395
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1251 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1248

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
ATG GAC GTA AGG CTG CAT CTG AAG CAA TTT GCT TTA CTG TGT TTT ATC        48
Met Asp Val Arg Leu His Leu Lys Gln Phe Ala Leu Leu Cys Phe Ile
  1               5                  10                  15

AGC TTG CTT CTG ACG CCT TGT GGA TTA GCC TGT GGT CCT GGT AGA GGT        96
Ser Leu Leu Leu Thr Pro Cys Gly Leu Ala Cys Gly Pro Gly Arg Gly
             20                  25                  30

TAT GGA AAA CGA AGA CAC CCA AAG AAA TTA ACC CCG TTG GCT TAC AAG       144
Tyr Gly Lys Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys
         35                  40                  45

CAA TTC ATC CCC AAC GTT GCT GAG AAA ACG CTT GGA GCC AGC GGC AAA       192
Gln Phe Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Lys
     50                  55                  60

TAC GAA GGC AAA ATC ACA AGG AAT TCA GAG AGA TTT AAA GAG CTG ATT       240
Tyr Glu Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Ile
 65                  70                  75                  80

CCG AAT TAT AAT CCC GAT ATC ATC TTT AAG GAC GAG GAA AAC ACA AAC       288
Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Asn
                 85                  90                  95

GCT GAC AGG CTG ATG ACC AAG CGC TGT AAG GAC AAG TTA AAT TCG TTG       336
Ala Asp Arg Leu Met Thr Lys Arg Cys Lys Asp Lys Leu Asn Ser Leu
            100                 105                 110

GCC ATA TCC GTC ATG AAC CAC TGG CCC GGC GTG AAA CTG CGC GTC ACT       384
Ala Ile Ser Val Met Asn His Trp Pro Gly Val Lys Leu Arg Val Thr
        115                 120                 125
```

GAA GGC TGG GAT GAG GAT GGT CAC CAT TTA GAA GAA TCT TTG CAC TAT 432
Glu Gly Trp Asp Glu Asp Gly His His Leu Glu Glu Ser Leu His Tyr
130 135 140

GAG GGA CGG GCA GTG GAC ATC ACT ACC TCA GAC AGG GAT AAA AGC AAG 480
Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Lys Ser Lys
145 150 155 160

TAT GGG ATG CTA TCC AGG CTT GCA GTG GAG GCA GGA TTC GAC TGG GTC 528
Tyr Gly Met Leu Ser Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val
165 170 175

TAT TAT GAA TCT AAA GCC CAC ATA CAC TGC TCT GTC AAA GCA GAA AAT 576
Tyr Tyr Glu Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn
180 185 190

TCA GTG GCT GCT AAA TCA GGA GGA TGT TTT CCT GGG TCT GGG ACG GTG 624
Ser Val Ala Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Gly Thr Val
195 200 205

ACA CTT GGT GAT GGG ACG AGG AAA CCC ATC AAA GAT CTT AAA GTG GGC 672
Thr Leu Gly Asp Gly Thr Arg Lys Pro Ile Lys Asp Leu Lys Val Gly
210 215 220

GAC CGG GTT TTG GCT GCA GAC GAG AAG GGA AAT GTC TTA ATA AGC GAC 720
Asp Arg Val Leu Ala Ala Asp Glu Lys Gly Asn Val Leu Ile Ser Asp
225 230 235 240

TTT ATT ATG TTT ATA GAC CAC GAT CCG ACA ACG AGA AGG CAA TTC ATC 768
Phe Ile Met Phe Ile Asp His Asp Pro Thr Thr Arg Arg Gln Phe Ile
245 250 255

GTC ATC GAG ACG TCA GAA CCT TTC ACC AAG CTC ACC CTC ACT GCC GCG 816
Val Ile Glu Thr Ser Glu Pro Phe Thr Lys Leu Thr Leu Thr Ala Ala
260 265 270

CAC CTA GTT TTC GTT GGA AAC TCT TCA GCA GCT TCG GGT ATA ACA GCA 864
His Leu Val Phe Val Gly Asn Ser Ser Ala Ala Ser Gly Ile Thr Ala
275 280 285

ACA TTT GCC AGC AAC GTG AAG CCT GGA GAT ACA GTT TTA GTG TGG GAA 912
Thr Phe Ala Ser Asn Val Lys Pro Gly Asp Thr Val Leu Val Trp Glu
290 295 300

GAC ACA TGC GAG AGC CTC AAG AGC GTT ACA GTG AAA AGG ATT TAC ACT 960
Asp Thr Cys Glu Ser Leu Lys Ser Val Thr Val Lys Arg Ile Tyr Thr
305 310 315 320

GAG GAG CAC GAG GGC TCT TTT GCG CCA GTC ACC GCG CAC GGA ACC ATA 1008
Glu Glu His Glu Gly Ser Phe Ala Pro Val Thr Ala His Gly Thr Ile
325 330 335

ATA GTG GAT CAG GTG TTG GCA TCG TGC TAC GCG GTC ATT GAG AAC CAC 1056
Ile Val Asp Gln Val Leu Ala Ser Cys Tyr Ala Val Ile Glu Asn His
340 345 350

AAA TGG GCA CAT TGG GCT TTT GCG CCG GTC AGG TTG TGT CAC AAG CTG 1104
Lys Trp Ala His Trp Ala Phe Ala Pro Val Arg Leu Cys His Lys Leu
355 360 365

```
ATG ACG TGG CTT TTT CCG GCT CGT GAA TCA AAC GTC AAT TTT CAG GAG       1152
Met Thr Trp Leu Phe Pro Ala Arg Glu Ser Asn Val Asn Phe Gln Glu
    370                 375                 380

GAT GGT ATC CAC TGG TAC TCA AAT ATG CTG TTT CAC ATC GGC TCT TGG       1200
Asp Gly Ile His Trp Tyr Ser Asn Met Leu Phe His Ile Gly Ser Trp
385                 390                 395                 400

CTG CTG GAC AGA GAC TCT TTC CAT CCA CTC GGG ATT TTA CAC TTA AGT       1248
Leu Leu Asp Arg Asp Ser Phe His Pro Leu Gly Ile Leu His Leu Ser
                405                 410                 415

TGA                                                                   1251
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 425 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Met Val Glu Met Leu Leu Leu Thr Arg Ile Leu Leu Val Gly Phe Ile
  1               5                  10                  15

Cys Ala Leu Leu Val Ser Ser Gly Leu Thr Cys Gly Pro Gly Arg Gly
             20                  25                  30

Ile Gly Lys Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys
         35                  40                  45

Gln Phe Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg
     50                  55                  60

Tyr Glu Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr
 65                  70                  75                  80

Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly
                 85                  90                  95

Ala Asp Arg Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu
            100                 105                 110

Ala Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg Val Thr
        115                 120                 125

Glu Gly Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr
    130                 135                 140

Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Ser Lys
145                 150                 155                 160

Tyr Gly Met Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val
                165                 170                 175
```

```
Tyr Tyr Glu Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn
            180                 185                 190

Ser Val Ala Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Thr Val
        195                 200                 205

His Leu Glu His Gly Gly Thr Lys Leu Val Lys Asp Leu Ser Pro Gly
    210                 215                 220

Asp Arg Val Leu Ala Ala Asp Ala Asp Gly Arg Leu Leu Tyr Ser Asp
225                 230                 235                 240

Phe Leu Thr Phe Leu Asp Arg Met Asp Ser Ser Arg Lys Leu Phe Tyr
                245                 250                 255

Val Ile Glu Thr Arg Gln Pro Arg Ala Arg Leu Leu Leu Thr Ala Ala
            260                 265                 270

His Leu Leu Phe Val Ala Pro Gln His Asn Gln Ser Glu Ala Thr Gly
        275                 280                 285

Ser Thr Ser Gly Gln Ala Leu Phe Ala Ser Asn Val Lys Pro Gly Gln
    290                 295                 300

Arg Val Tyr Val Leu Gly Glu Gly Gly Gln Gln Leu Leu Pro Ala Ser
305                 310                 315                 320

Val His Ser Val Ser Leu Arg Glu Glu Ala Ser Gly Ala Tyr Ala Pro
                325                 330                 335

Leu Thr Ala Gln Gly Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys
            340                 345                 350

Tyr Ala Val Ile Glu Glu His Ser Trp Ala His Trp Ala Phe Ala Pro
        355                 360                 365

Phe Arg Leu Ala Gln Gly Leu Leu Ala Ala Leu Cys Pro Asp Gly Ala
    370                 375                 380

Ile Pro Thr Ala Ala Thr Thr Thr Thr Gly Ile His Trp Tyr Ser Arg
385                 390                 395                 400

Leu Leu Tyr Arg Ile Gly Ser Trp Val Leu Asp Gly Asp Ala Leu His
                405                 410                 415

Pro Leu Gly Met Val Ala Pro Ala Ser
            420                 425
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 396 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Met Ala Leu Pro Ala Ser Leu Leu Pro Leu Cys Cys Leu Ala Leu Leu
1 5 10 15

Ala Leu Ser Ala Gln Ser Cys Gly Pro Gly Arg Gly Pro Val Gly Arg
20 25 30

Arg Arg Tyr Val Arg Lys Gln Leu Val Pro Leu Leu Tyr Lys Gln Phe
35 40 45

Val Pro Ser Met Pro Glu Arg Thr Leu Gly Ala Ser Gly Pro Ala Glu
50 55 60

Gly Arg Val Thr Arg Gly Ser Glu Arg Phe Arg Asp Leu Val Pro Asn
65 70 75 80

Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Ser Gly Ala Asp
85 90 95

Arg Leu Met Thr Glu Arg Cys Lys Glu Arg Val Asn Ala Leu Ala Ile
100 105 110

Ala Val Met Asn Met Trp Pro Gly Val Arg Leu Arg Val Thr Glu Gly
115 120 125

Trp Asp Glu Asp Gly His His Ala Gln Asp Ser Leu His Tyr Glu Gly
130 135 140

Arg Ala Leu Asp Ile Thr Thr Ser Asp Arg Asp Arg Asn Lys Tyr Gly
145 150 155 160

Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr
165 170 175

Glu Ser Arg Asn His Ile His Val Ser Val Lys Ala Asp Asn Ser Leu
180 185 190

Ala Val Arg Ala Gly Gly Cys Phe Pro Gly Asn Ala Thr Val Arg Leu
195 200 205

Arg Ser Gly Glu Arg Lys Gly Leu Arg Glu Leu His Arg Gly Asp Trp
210 215 220

Val Leu Ala Ala Asp Ala Ala Gly Arg Val Val Pro Thr Pro Val Leu
225 230 235 240

Leu Phe Leu Asp Arg Asp Leu Gln Arg Arg Ala Ser Phe Val Ala Val
245 250 255

Glu Thr Glu Arg Pro Pro Arg Lys Leu Leu Leu Thr Pro Trp His Leu
260 265 270

Val Phe Ala Ala Arg Gly Pro Ala Pro Ala Pro Gly Asp Phe Ala Pro
275 280 285

Val Phe Ala Arg Arg Leu Arg Ala Gly Asp Ser Val Leu Ala Pro Gly
290 295 300

Gly Asp Ala Leu Gln Pro Ala Arg Val Ala Arg Val Ala Arg Glu Glu
305 310 315 320

```
Ala Val Gly Val Phe Ala Pro Leu Thr Ala His Gly Thr Leu Leu Val
                325                 330                 335

Asn Asp Val Leu Ala Ser Cys Tyr Ala Val Leu Glu Ser His Gln Trp
            340                 345                 350

Ala His Arg Ala Phe Ala Pro Leu Arg Leu Leu His Ala Leu Gly Ala
        355                 360                 365

Leu Leu Pro Gly Gly Ala Val Gln Pro Thr Gly Met His Trp Tyr Ser
    370                 375                 380

Arg Leu Leu Tyr Arg Leu Ala Glu Glu Leu Met Gly
385                 390                 395
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 411 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Met Ser Pro Ala Trp Leu Arg Pro Arg Leu Arg Phe Cys Leu Phe Leu
  1               5                  10                  15

Leu Leu Leu Leu Leu Val Pro Ala Ala Arg Gly Cys Gly Pro Gly Arg
            20                  25                  30

Val Val Gly Ser Arg Arg Arg Pro Pro Arg Lys Leu Val Pro Leu Ala
        35                  40                  45

Tyr Lys Gln Phe Ser Pro Asn Val Pro Glu Lys Thr Leu Gly Ala Ser
    50                  55                  60

Gly Arg Tyr Glu Gly Lys Ile Ala Arg Ser Ser Glu Arg Phe Lys Glu
 65                  70                  75                  80

Leu Thr Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn
                85                  90                  95

Thr Gly Ala Asp Arg Leu Met Thr Gln Arg Cys Lys Asp Arg Leu Asn
            100                 105                 110

Ser Leu Ala Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg
        115                 120                 125

Val Thr Glu Gly Arg Asp Glu Asp Gly His His Ser Glu Glu Ser Leu
    130                 135                 140

His Tyr Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg
145                 150                 155                 160

Asn Lys Tyr Gly Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp
                165                 170                 175
```

Trp Val Tyr Tyr Glu Ser Lys Ala His Val His Cys Ser Val Lys Ser
180 185 190

Glu His Ser Ala Ala Ala Lys Thr Gly Gly Cys Phe Pro Ala Gly Ala
195 200 205

Gln Val Arg Leu Glu Asn Gly Glu Arg Val Ala Leu Ser Ala Val Lys
210 215 220

Pro Gly Asp Arg Val Leu Ala Met Gly Glu Asp Gly Thr Pro Thr Phe
225 230 235 240

Ser Asp Val Leu Ile Phe Leu Asp Arg Glu Pro Asn Arg Leu Arg Ala
245 250 255

Phe Gln Val Ile Glu Thr Gln Asp Pro Pro Arg Arg Leu Ala Leu Thr
260 265 270

Pro Ala His Leu Leu Phe Ile Ala Asp Asn His Thr Glu Pro Ala Ala
275 280 285

His Phe Arg Ala Thr Phe Ala Ser His Val Gln Pro Gly Gln Tyr Val
290 295 300

Leu Val Ser Gly Val Pro Gly Leu Gln Pro Ala Arg Val Ala Ala Val
305 310 315 320

Ser Thr His Val Ala Leu Gly Ser Tyr Ala Pro Leu Thr Arg His Gly
325 330 335

Thr Leu Val Val Glu Asp Val Val Ala Ser Cys Phe Ala Ala Val Ala
340 345 350

Asp His His Leu Ala Gln Leu Ala Phe Trp Pro Leu Arg Leu Phe Pro
355 360 365

Ser Leu Ala Trp Gly Ser Trp Thr Pro Ser Glu Gly Val His Ser Tyr
370 375 380

Pro Gln Met Leu Tyr Arg Leu Gly Arg Leu Leu Leu Glu Glu Ser Thr
385 390 395 400

Phe His Pro Leu Gly Met Ser Gly Ala Gly Ser
405 410

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 437 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
Met Leu Leu Leu Leu Ala Arg Cys Phe Leu Val Ile Leu Ala Ser Ser
1               5                   10                  15

Leu Leu Val Cys Pro Gly Leu Ala Cys Gly Pro Gly Arg Gly Phe Gly
            20                  25                  30

Lys Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe
        35                  40                  45

Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu
    50                  55                  60

Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn
65                  70                  75                  80

Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp
                85                  90                  95

Arg Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu Ala Ile
            100                 105                 110

Ser Val Met Asn Gln Trp Pro Gly Val Arg Leu Arg Val Thr Glu Gly
        115                 120                 125

Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr Glu Gly
    130                 135                 140

Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Ser Lys Tyr Gly
145                 150                 155                 160

Met Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr
                165                 170                 175

Glu Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn Ser Val
            180                 185                 190

Ala Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Thr Val His Leu
        195                 200                 205

Glu Gln Gly Gly Thr Lys Leu Val Lys Asp Leu Arg Pro Gly Asp Arg
    210                 215                 220

Val Leu Ala Ala Asp Asp Gln Gly Arg Leu Leu Tyr Ser Asp Phe Leu
225                 230                 235                 240

Thr Phe Leu Asp Arg Asp Glu Gly Ala Lys Lys Val Phe Tyr Val Ile
                245                 250                 255

Glu Thr Leu Glu Pro Arg Glu Arg Leu Leu Leu Thr Ala Ala His Leu
            260                 265                 270

Leu Phe Val Ala Pro His Asn Asp Ser Gly Pro Thr Pro Gly Pro Ser
        275                 280                 285

Ala Leu Phe Ala Ser Arg Val Arg Pro Gly Gln Arg Val Tyr Val Val
    290                 295                 300

Ala Glu Arg Gly Gly Asp Arg Arg Leu Leu Pro Ala Ala Val His Ser
305                 310                 315                 320
```

Val Thr Leu Arg Glu Glu Glu Ala Gly Ala Tyr Ala Pro Leu Thr Ala
325 330 335

His Gly Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys Tyr Ala Val
340 345 350

Ile Glu Glu His Ser Trp Ala His Arg Ala Phe Ala Pro Phe Arg Leu
355 360 365

Ala His Ala Leu Leu Ala Ala Leu Ala Pro Ala Arg Thr Asp Gly Gly
370 375 380

Gly Gly Gly Ser Ile Pro Ala Ala Gln Ser Ala Thr Glu Ala Arg Gly
385 390 395 400

Ala Glu Pro Thr Ala Gly Ile His Trp Tyr Ser Gln Leu Leu Tyr His
405 410 415

Ile Gly Thr Trp Leu Leu Asp Ser Glu Thr Met His Pro Leu Gly Met
420 425 430

Ala Val Lys Ser Ser
435

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 418 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Met Arg Leu Leu Thr Arg Val Leu Leu Val Ser Leu Leu Thr Leu Ser
1 5 10 15

Leu Val Val Ser Gly Leu Ala Cys Gly Pro Gly Arg Gly Tyr Gly Arg
20 25 30

Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe Ile
35 40 45

Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu Gly
50 55 60

Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn Tyr
65 70 75 80

Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp Arg
85 90 95

Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ser Leu Ala Ile Ser
100 105 110

Val Met Asn His Trp Pro Gly Val Lys Leu Arg Val Thr Glu Gly Trp
115 120 125

```
Asp Glu Asp Gly His His Phe Glu Glu Ser Leu His Tyr Glu Gly Arg
    130                 135                 140

Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Lys Ser Lys Tyr Gly Thr
145                 150                 155                 160

Leu Ser Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr Glu
                165                 170                 175

Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn Ser Val Ala
            180                 185                 190

Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Leu Val Ser Leu Gln
        195                 200                 205

Asp Gly Gly Gln Lys Ala Val Lys Asp Leu Asn Pro Gly Asp Lys Val
    210                 215                 220

Leu Ala Ala Asp Ser Ala Gly Asn Leu Val Phe Ser Asp Phe Ile Met
225                 230                 235                 240

Phe Thr Asp Arg Asp Ser Thr Thr Arg Arg Val Phe Tyr Val Ile Glu
                245                 250                 255

Thr Gln Glu Pro Val Glu Lys Ile Thr Leu Thr Ala Ala His Leu Leu
            260                 265                 270

Phe Val Leu Asp Asn Ser Thr Glu Asp Leu His Thr Met Thr Ala Ala
        275                 280                 285

Tyr Ala Ser Ser Val Arg Ala Gly Gln Lys Val Met Val Val Asp Asp
    290                 295                 300

Ser Gly Gln Leu Lys Ser Val Ile Val Gln Arg Ile Tyr Thr Glu Glu
305                 310                 315                 320

Gln Arg Gly Ser Phe Ala Pro Val Thr Ala His Gly Thr Ile Val Val
                325                 330                 335

Asp Arg Ile Leu Ala Ser Cys Tyr Ala Val Ile Glu Asp Gln Gly Leu
            340                 345                 350

Ala His Leu Ala Phe Ala Pro Ala Arg Leu Tyr Tyr Tyr Val Ser Ser
        355                 360                 365

Phe Leu Ser Pro Lys Thr Pro Ala Val Gly Pro Met Arg Leu Tyr Asn
    370                 375                 380

Arg Arg Gly Ser Thr Gly Thr Pro Gly Ser Cys His Gln Met Gly Thr
385                 390                 395                 400

Trp Leu Leu Asp Ser Asn Met Leu His Pro Leu Gly Met Ser Val Asn
                405                 410                 415

Ser Ser
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 475 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
Met Leu Leu Leu Ala Arg Cys Leu Leu Leu Val Leu Val Ser Ser Leu
 1               5                  10                  15

Leu Val Cys Ser Gly Leu Ala Cys Gly Pro Gly Arg Gly Phe Gly Lys
            20                  25                  30

Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe Ile
        35                  40                  45

Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu Gly
    50                  55                  60

Lys Ile Ser Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn Tyr
65                  70                  75                  80

Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp Arg
                85                  90                  95

Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu Ala Ile Ser
            100                 105                 110

Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg Val Thr Glu Gly Trp
        115                 120                 125

Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr Glu Gly Arg
    130                 135                 140

Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Ser Lys Tyr Gly Met
145                 150                 155                 160

Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr Glu
                165                 170                 175

Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn Ser Val Ala
            180                 185                 190

Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Thr Val His Leu Glu
        195                 200                 205

Gln Gly Gly Thr Lys Leu Val Lys Asp Leu Ser Pro Gly Asp Arg Val
    210                 215                 220

Leu Ala Ala Asp Asp Gln Gly Arg Leu Leu Tyr Ser Asp Phe Leu Thr
225                 230                 235                 240

Phe Leu Asp Arg Asp Asp Gly Ala Lys Lys Val Phe Tyr Val Ile Glu
                245                 250                 255

Thr Arg Glu Pro Arg Glu Arg Leu Leu Leu Thr Ala Ala His Leu Leu
            260                 265                 270
```

Phe Val Ala Pro His Asn Asp Ser Ala Thr Gly Glu Pro Glu Ala Ser
275 280 285

Ser Gly Ser Gly Pro Pro Ser Gly Gly Ala Leu Gly Pro Arg Ala Leu
290 295 300

Phe Ala Ser Arg Val Arg Pro Gly Gln Arg Val Tyr Val Val Ala Glu
305 310 315 320

Arg Asp Gly Asp Arg Arg Leu Leu Pro Ala Ala Val His Ser Val Thr
325 330 335

Leu Ser Glu Glu Ala Ala Gly Ala Tyr Ala Pro Leu Thr Ala Gln Gly
340 345 350

Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys Tyr Ala Val Ile Glu
355 360 365

Glu His Ser Trp Ala His Arg Ala Phe Ala Pro Phe Arg Leu Ala His
370 375 380

Ala Leu Leu Ala Ala Leu Ala Pro Ala Arg Thr Asp Arg Gly Gly Asp
385 390 395 400

Ser Gly Gly Gly Asp Arg Gly Gly Gly Gly Gly Arg Val Ala Leu Thr
405 410 415

Ala Pro Gly Ala Ala Asp Ala Pro Gly Ala Gly Ala Thr Ala Gly Ile
420 425 430

His Trp Tyr Ser Gln Leu Leu Tyr Gln Ile Gly Thr Trp Leu Leu Asp
435 440 445

Ser Glu Ala Leu His Pro Leu Gly Met Ala Val Lys Ser Ser Xaa Ser
450 455 460

Arg Gly Ala Gly Gly Gly Ala Arg Glu Gly Ala
465 470 475

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 411 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Met Ser Pro Ala Arg Leu Arg Pro Arg Leu His Phe Cys Leu Val Leu
1 5 10 15

Leu Leu Leu Leu Val Val Pro Ala Ala Trp Gly Cys Gly Pro Gly Arg
20 25 30

Val Val Gly Ser Arg Arg Arg Pro Pro Arg Lys Leu Val Pro Leu Ala
35 40 45

Tyr Lys Gln Phe Ser Pro Asn Val Pro Glu Lys Thr Leu Gly Ala Ser
    50   55   60

Gly Arg Tyr Glu Gly Lys Ile Ala Arg Ser Ser Glu Arg Phe Lys Glu
65   70   75   80

Leu Thr Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn
                85   90   95

Thr Gly Ala Asp Arg Leu Met Thr Gln Arg Cys Lys Asp Arg Leu Asn
            100   105   110

Ser Leu Ala Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg
        115   120   125

Val Thr Glu Gly Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu
    130   135   140

His Tyr Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg
145   150   155   160

Asn Lys Tyr Gly Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp
                165   170   175

Trp Val Tyr Tyr Glu Ser Lys Ala His Val His Cys Ser Val Lys Ser
            180   185   190

Glu His Ser Ala Ala Ala Lys Thr Gly Gly Cys Phe Pro Ala Gly Ala
        195   200   205

Gln Val Arg Leu Glu Ser Gly Ala Arg Val Ala Leu Ser Ala Val Arg
    210   215   220

Pro Gly Asp Arg Val Leu Ala Met Gly Glu Asp Gly Ser Pro Thr Phe
225   230   235   240

Ser Asp Val Leu Ile Phe Leu Asp Arg Glu Pro His Arg Leu Arg Ala
                245   250   255

Phe Gln Val Ile Glu Thr Gln Asp Pro Pro Arg Arg Leu Ala Leu Thr
            260   265   270

Pro Ala His Leu Leu Phe Thr Ala Asp Asn His Thr Glu Pro Ala Ala
        275   280   285

Arg Phe Arg Ala Thr Phe Ala Ser His Val Gln Pro Gly Gln Tyr Val
    290   295   300

Leu Val Ala Gly Val Pro Gly Leu Gln Pro Ala Arg Val Ala Ala Val
305   310   315   320

Ser Thr His Val Ala Leu Gly Ala Tyr Ala Pro Leu Thr Lys His Gly
                325   330   335

Thr Leu Val Val Glu Asp Val Val Ala Ser Cys Phe Ala Ala Val Ala
            340   345   350

Asp His His Leu Ala Gln Leu Ala Phe Trp Pro Leu Arg Leu Phe His
        355   360   365

Ser Leu Ala Trp Gly Ser Trp Thr Pro Gly Glu Gly Val His Trp Tyr
370 375 380

Pro Gln Leu Leu Tyr Arg Leu Gly Arg Leu Leu Leu Glu Glu Gly Ser
385 390 395 400

Phe His Pro Leu Gly Met Ser Gly Ala Gly Ser
405 410

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 396 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Met Ala Leu Leu Thr Asn Leu Leu Pro Leu Cys Cys Leu Ala Leu Leu
1 5 10 15

Ala Leu Pro Ala Gln Ser Cys Gly Pro Gly Arg Gly Pro Val Gly Arg
20 25 30

Arg Arg Tyr Ala Arg Lys Gln Leu Val Pro Leu Leu Tyr Lys Gln Phe
35 40 45

Val Pro Gly Val Pro Glu Arg Thr Leu Gly Ala Ser Gly Pro Ala Glu
50 55 60

Gly Arg Val Ala Arg Gly Ser Glu Arg Phe Arg Asp Leu Val Pro Asn
65 70 75 80

Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Ser Gly Ala Asp
85 90 95

Arg Leu Met Thr Glu Arg Cys Lys Glu Arg Val Asn Ala Leu Ala Ile
100 105 110

Ala Val Met Asn Met Trp Pro Gly Val Arg Leu Arg Val Thr Glu Gly
115 120 125

Trp Asp Glu Asp Gly His His Ala Gln Asp Ser Leu His Tyr Glu Gly
130 135 140

Arg Ala Leu Asp Ile Thr Thr Ser Asp Arg Asp Arg Asn Lys Tyr Gly
145 150 155 160

Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr
165 170 175

Glu Ser Arg Asn His Val His Val Ser Val Lys Ala Asp Asn Ser Leu
180 185 190

Ala Val Arg Ala Gly Gly Cys Phe Pro Gly Asn Ala Thr Val Arg Leu
195 200 205

Trp Ser Gly Glu Arg Lys Gly Leu Arg Glu Leu His Arg Gly Asp Trp
210 215 220

```
Val Leu Ala Ala Asp Ala Ser Gly Arg Val Val Pro Thr Pro Val Leu
225                 230                 235                 240

Leu Phe Leu Asp Arg Asp Leu Gln Arg Arg Ala Ser Phe Val Ala Val
                245                 250                 255

Glu Thr Glu Trp Pro Pro Arg Lys Leu Leu Leu Thr Pro Trp His Leu
            260                 265                 270

Val Phe Ala Ala Arg Gly Pro Ala Pro Ala Pro Gly Asp Phe Ala Pro
        275                 280                 285

Val Phe Ala Arg Arg Leu Arg Ala Gly Asp Ser Val Leu Ala Pro Gly
    290                 295                 300

Gly Asp Ala Leu Arg Pro Ala Arg Val Ala Arg Val Ala Arg Glu Glu
305                 310                 315                 320

Ala Val Gly Val Phe Ala Pro Leu Thr Ala His Gly Thr Leu Leu Val
                325                 330                 335

Asn Asp Val Leu Ala Ser Cys Tyr Ala Val Leu Glu Ser His Gln Trp
            340                 345                 350

Ala His Arg Ala Phe Ala Pro Leu Arg Leu Leu His Ala Leu Gly Ala
        355                 360                 365

Leu Leu Pro Gly Gly Ala Val Gln Pro Thr Gly Met His Trp Tyr Ser
    370                 375                 380

Arg Leu Leu Tyr Arg Leu Ala Glu Glu Leu Leu Gly
385                 390                 395
```

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 416 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

```
Met Asp Val Arg Leu His Leu Lys Gln Phe Ala Leu Leu Cys Phe Ile
  1               5                  10                  15

Ser Leu Leu Leu Thr Pro Cys Gly Leu Ala Cys Gly Pro Gly Arg Gly
             20                  25                  30

Tyr Gly Lys Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys
         35                  40                  45

Gln Phe Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Lys
     50                  55                  60

Tyr Glu Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Ile
 65                  70                  75                  80
```

```
Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Asn
                85                  90                  95

Ala Asp Arg Leu Met Thr Lys Arg Cys Lys Asp Lys Leu Asn Ser Leu
            100                 105                 110

Ala Ile Ser Val Met Asn His Trp Pro Gly Val Lys Leu Arg Val Thr
        115                 120                 125

Glu Gly Trp Asp Glu Asp Gly His His Leu Glu Glu Ser Leu His Tyr
    130                 135                 140

Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Lys Ser Lys
145                 150                 155                 160

Tyr Gly Met Leu Ser Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val
                165                 170                 175

Tyr Tyr Glu Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn
            180                 185                 190

Ser Val Ala Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Gly Thr Val
        195                 200                 205

Thr Leu Gly Asp Gly Thr Arg Lys Pro Ile Lys Asp Leu Lys Val Gly
    210                 215                 220

Asp Arg Val Leu Ala Ala Asp Glu Lys Gly Asn Val Leu Ile Ser Asp
225                 230                 235                 240

Phe Ile Met Phe Ile Asp His Asp Pro Thr Thr Arg Arg Gln Phe Ile
                245                 250                 255

Val Ile Glu Thr Ser Glu Pro Phe Thr Lys Leu Thr Leu Thr Ala Ala
            260                 265                 270

His Leu Val Phe Val Gly Asn Ser Ser Ala Ala Ser Gly Ile Thr Ala
        275                 280                 285

Thr Phe Ala Ser Asn Val Lys Pro Gly Asp Thr Val Leu Val Trp Glu
    290                 295                 300

Asp Thr Cys Glu Ser Leu Lys Ser Val Thr Val Lys Arg Ile Tyr Thr
305                 310                 315                 320

Glu Glu His Glu Gly Ser Phe Ala Pro Val Thr Ala His Gly Thr Ile
                325                 330                 335

Ile Val Asp Gln Val Leu Ala Ser Cys Tyr Ala Val Ile Glu Asn His
            340                 345                 350

Lys Trp Ala His Trp Ala Phe Ala Pro Val Arg Leu Cys His Lys Leu
        355                 360                 365

Met Thr Trp Leu Phe Pro Ala Arg Glu Ser Asn Val Asn Phe Gln Glu
    370                 375                 380

Asp Gly Ile His Trp Tyr Ser Asn Met Leu Phe His Ile Gly Ser Trp
385                 390                 395                 400
```

Leu Leu Asp Arg Asp Ser Phe His Pro Leu Gly Ile Leu His Leu Ser
405 410 415

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1416 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1413

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

ATG GAT AAC CAC AGC TCA GTG CCT TGG GCC AGT GCC GCC AGT GTC ACC 48
Met Asp Asn His Ser Ser Val Pro Trp Ala Ser Ala Ala Ser Val Thr
1 5 10 15

TGT CTC TCC CTG GGA TGC CAA ATG CCA CAG TTC CAG TTC CAG TTC CAG 96
Cys Leu Ser Leu Gly Cys Gln Met Pro Gln Phe Gln Phe Gln Phe Gln
20 25 30

CTC CAA ATC CGC AGC GAG CTC CAT CTC CGC AAG CCC GCA AGA AGA ACG 144
Leu Gln Ile Arg Ser Glu Leu His Leu Arg Lys Pro Ala Arg Arg Thr
35 40 45

CAA ACG ATG CGC CAC ATT GCG CAT ACG CAG CGT TGC CTC AGC AGG CTG 192
Gln Thr Met Arg His Ile Ala His Thr Gln Arg Cys Leu Ser Arg Leu
50 55 60

ACC TCT CTG GTG GCC CTG CTG CTG ATC GTC TTG CCG ATG GTC TTT AGC 240
Thr Ser Leu Val Ala Leu Leu Leu Ile Val Leu Pro Met Val Phe Ser
65 70 75 80

CCG GCT CAC AGC TGC GGT CCT GGC CGA GGA TTG GGT CGT CAT AGG GCG 288
Pro Ala His Ser Cys Gly Pro Gly Arg Gly Leu Gly Arg His Arg Ala
85 90 95

CGC AAC CTG TAT CCG CTG GTC CTC AAG CAG ACA ATT CCC AAT CTA TCC 336
Arg Asn Leu Tyr Pro Leu Val Leu Lys Gln Thr Ile Pro Asn Leu Ser
100 105 110

GAG TAC ACG AAC AGC GCC TCC GGA CCT CTG GAG GGT GTG ATC CGT CGG 384
Glu Tyr Thr Asn Ser Ala Ser Gly Pro Leu Glu Gly Val Ile Arg Arg
115 120 125

GAT TCG CCC AAA TTC AAG GAC CTC GTG CCC AAC TAC AAC AGG GAC ATC 432
Asp Ser Pro Lys Phe Lys Asp Leu Val Pro Asn Tyr Asn Arg Asp Ile
130 135 140

CTT TTC CGT GAC GAG GAA GGC ACC GGA GCG GAT GGC TTG ATG AGC AAG 480
Leu Phe Arg Asp Glu Glu Gly Thr Gly Ala Asp Gly Leu Met Ser Lys
145 150 155 160

```
CGC TGC AAG GAG AAG CTA AAC GTG CTG GCC TAC TCG GTG ATG AAC GAA        528
Arg Cys Lys Glu Lys Leu Asn Val Leu Ala Tyr Ser Val Met Asn Glu
                165                 170                 175

TGG CCC GGC ATC CGG CTG CTG GTC ACC GAG AGC TGG GAC GAG GAC TAC        576
Trp Pro Gly Ile Arg Leu Leu Val Thr Glu Ser Trp Asp Glu Asp Tyr
            180                 185                 190

CAT CAC GGC CAG GAG TCG CTC CAC TAC GAG GGC CGA GCG GTG ACC ATT        624
His His Gly Gln Glu Ser Leu His Tyr Glu Gly Arg Ala Val Thr Ile
        195                 200                 205

GCC ACC TCC GAT CGC GAC CAG TCC AAA TAC GGC ATG CTC GCT CGC CTG        672
Ala Thr Ser Asp Arg Asp Gln Ser Lys Tyr Gly Met Leu Ala Arg Leu
    210                 215                 220

GCC GTC GAG GCT GGA TTC GAT TGG GTC TCC TAC GTC AGC AGG CGC CAC        720
Ala Val Glu Ala Gly Phe Asp Trp Val Ser Tyr Val Ser Arg Arg His
225                 230                 235                 240

ATC TAC TGC TCC GTC AAG TCA GAT TCG TCG ATC AGT TCC CAC GTG CAC        768
Ile Tyr Cys Ser Val Lys Ser Asp Ser Ser Ile Ser Ser His Val His
                245                 250                 255

GGC TGC TTC ACG CCG GAG AGC ACA GCG CTG CTG GAG AGT GGA GTC CGG        816
Gly Cys Phe Thr Pro Glu Ser Thr Ala Leu Leu Glu Ser Gly Val Arg
            260                 265                 270

AAG CCG CTC GGC GAG CTC TCT ATC GGA GAT CGT GTT TTG AGC ATG ACC        864
Lys Pro Leu Gly Glu Leu Ser Ile Gly Asp Arg Val Leu Ser Met Thr
        275                 280                 285

GCC AAC GGA CAG GCC GTC TAC AGC GAA GTG ATC CTC TTC ATG GAC CGC        912
Ala Asn Gly Gln Ala Val Tyr Ser Glu Val Ile Leu Phe Met Asp Arg
    290                 295                 300

AAC CTC GAG CAG ATG CAA AAC TTT GTG CAG CTG CAC ACG GAC GGT GGA        960
Asn Leu Glu Gln Met Gln Asn Phe Val Gln Leu His Thr Asp Gly Gly
305                 310                 315                 320

GCA GTG CTC ACG GTG ACG CCG GCT CAC CTG GTT AGC GTT TGG CAG CCG       1008
Ala Val Leu Thr Val Thr Pro Ala His Leu Val Ser Val Trp Gln Pro
                325                 330                 335

GAG AGC CAG AAG CTC ACG TTT GTG TTT GCG CAT CGC ATC GAG GAG AAG       1056
Glu Ser Gln Lys Leu Thr Phe Val Phe Ala His Arg Ile Glu Glu Lys
            340                 345                 350

AAC CAG GTG CTC GTA CGG GAT GTG GAG ACG GGC GAG CTG AGG CCC CAG       1104
Asn Gln Val Leu Val Arg Asp Val Glu Thr Gly Glu Leu Arg Pro Gln
        355                 360                 365

CGA GTG GTC AAG TTG GGC AGT GTG CGC AGT AAG GGC GTG GTC GCG CCG       1152
Arg Val Val Lys Leu Gly Ser Val Arg Ser Lys Gly Val Val Ala Pro
    370                 375                 380

CTG ACC CGC GAG GGC ACC ATT GTG GTC AAC TCG GTG GCC GCC AGT TGC       1200
Leu Thr Arg Glu Gly Thr Ile Val Val Asn Ser Val Ala Ala Ser Cys
385                 390                 395                 400
```

```
TAT GCG GTG ATC AAC AGT CAG TCG CTG GCC CAC TGG GGA CTG GCT CCC        1248
Tyr Ala Val Ile Asn Ser Gln Ser Leu Ala His Trp Gly Leu Ala Pro
                405                 410                 415

ATG CGC CTG CTG TCC ACG CTG GAG GCG TGG CTG CCC GCC AAG GAG CAG        1296
Met Arg Leu Leu Ser Thr Leu Glu Ala Trp Leu Pro Ala Lys Glu Gln
            420                 425                 430

TTG CAC AGT TCG CCG AAG GTG GTG AGC TCG GCG CAG CAG CAG AAT GGC        1344
Leu His Ser Ser Pro Lys Val Val Ser Ser Ala Gln Gln Gln Asn Gly
        435                 440                 445

ATC CAT TGG TAT GCC AAT GCG CTC TAC AAG GTC AAG GAC TAC GTG CTG        1392
Ile His Trp Tyr Ala Asn Ala Leu Tyr Lys Val Lys Asp Tyr Val Leu
    450                 455                 460

CCG CAG AGC TGG CGC CAC GAT TGA                                        1416
Pro Gln Ser Trp Arg His Asp
465                 470
```

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 471 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```
Met Asp Asn His Ser Ser Val Pro Trp Ala Ser Ala Ala Ser Val Thr
  1               5                  10                  15

Cys Leu Ser Leu Gly Cys Gln Met Pro Gln Phe Gln Phe Gln Phe Gln
            20                  25                  30

Leu Gln Ile Arg Ser Glu Leu His Leu Arg Lys Pro Ala Arg Arg Thr
        35                  40                  45

Gln Thr Met Arg His Ile Ala His Thr Gln Arg Cys Leu Ser Arg Leu
    50                  55                  60

Thr Ser Leu Val Ala Leu Leu Leu Ile Val Leu Pro Met Val Phe Ser
 65                  70                  75                  80

Pro Ala His Ser Cys Gly Pro Gly Arg Gly Leu Gly Arg His Arg Ala
                 85                  90                  95

Arg Asn Leu Tyr Pro Leu Val Leu Lys Gln Thr Ile Pro Asn Leu Ser
            100                 105                 110

Glu Tyr Thr Asn Ser Ala Ser Gly Pro Leu Glu Gly Val Ile Arg Arg
        115                 120                 125

Asp Ser Pro Lys Phe Lys Asp Leu Val Pro Asn Tyr Asn Arg Asp Ile
    130                 135                 140

Leu Phe Arg Asp Glu Glu Gly Thr Gly Ala Asp Gly Leu Met Ser Lys
145                 150                 155                 160
```

Arg Cys Lys Glu Lys Leu Asn Val Leu Ala Tyr Ser Val Met Asn Glu
                165   170   175

Trp Pro Gly Ile Arg Leu Leu Val Thr Glu Ser Trp Asp Glu Asp Tyr
            180   185   190

His His Gly Gln Glu Ser Leu His Tyr Glu Gly Arg Ala Val Thr Ile
        195   200   205

Ala Thr Ser Asp Arg Asp Gln Ser Lys Tyr Gly Met Leu Ala Arg Leu
    210   215   220

Ala Val Glu Ala Gly Phe Asp Trp Val Ser Tyr Val Ser Arg Arg His
225   230   235   240

Ile Tyr Cys Ser Val Lys Ser Asp Ser Ser Ile Ser Ser His Val His
                245   250   255

Gly Cys Phe Thr Pro Glu Ser Thr Ala Leu Leu Glu Ser Gly Val Arg
            260   265   270

Lys Pro Leu Gly Glu Leu Ser Ile Gly Asp Arg Val Leu Ser Met Thr
        275   280   285

Ala Asn Gly Gln Ala Val Tyr Ser Glu Val Ile Leu Phe Met Asp Arg
    290   295   300

Asn Leu Glu Gln Met Gln Asn Phe Val Gln Leu His Thr Asp Gly Gly
305   310   315   320

Ala Val Leu Thr Val Thr Pro Ala His Leu Val Ser Val Trp Gln Pro
                325   330   335

Glu Ser Gln Lys Leu Thr Phe Val Phe Ala His Arg Ile Glu Glu Lys
            340   345   350

Asn Gln Val Leu Val Arg Asp Val Glu Thr Gly Glu Leu Arg Pro Gln
        355   360   365

Arg Val Val Lys Leu Gly Ser Val Arg Ser Lys Gly Val Val Ala Pro
    370   375   380

Leu Thr Arg Glu Gly Thr Ile Val Val Asn Ser Val Ala Ala Ser Cys
385   390   395   400

Tyr Ala Val Ile Asn Ser Gln Ser Leu Ala His Trp Gly Leu Ala Pro
                405   410   415

Met Arg Leu Leu Ser Thr Leu Glu Ala Trp Leu Pro Ala Lys Glu Gln
            420   425   430

Leu His Ser Ser Pro Lys Val Val Ser Ser Ala Gln Gln Gln Asn Gly
        435   440   445

Ile His Trp Tyr Ala Asn Ala Leu Tyr Lys Val Lys Asp Tyr Val Leu
    450   455   460

Pro Gln Ser Trp Arg His Asp
465   470

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 221 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

```
Cys Gly Pro Gly Arg Gly Xaa Gly Xaa Arg Arg His Pro Lys Lys Leu
1               5                   10                  15

Thr Pro Leu Ala Tyr Lys Gln Phe Ile Pro Asn Val Ala Glu Lys Thr
            20                  25                  30

Leu Gly Ala Ser Gly Arg Tyr Glu Gly Lys Ile Xaa Arg Asn Ser Glu
        35                  40                  45

Arg Phe Lys Glu Leu Thr Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys
    50                  55                  60

Asp Glu Glu Asn Thr Gly Ala Asp Arg Leu Met Thr Gln Arg Cys Lys
65                  70                  75                  80

Asp Lys Leu Asn Xaa Leu Ala Ile Ser Val Met Asn Xaa Trp Pro Gly
                85                  90                  95

Val Xaa Leu Arg Val Thr Glu Gly Trp Asp Glu Asp Gly His His Xaa
            100                 105                 110

Glu Glu Ser Leu His Tyr Glu Gly Arg Ala Val Asp Ile Thr Thr Ser
        115                 120                 125

Asp Arg Asp Xaa Ser Lys Tyr Gly Xaa Leu Xaa Arg Leu Ala Val Glu
    130                 135                 140

Ala Gly Phe Asp Trp Val Tyr Tyr Glu Ser Lys Ala His Ile His Cys
145                 150                 155                 160

Ser Val Lys Ala Glu Asn Ser Val Ala Ala Lys Ser Gly Gly Cys Phe
                165                 170                 175

Pro Gly Ser Ala Xaa Val Xaa Leu Xaa Xaa Gly Gly Xaa Lys Xaa Val
            180                 185                 190

Lys Asp Leu Xaa Pro Gly Asp Xaa Val Leu Ala Ala Asp Xaa Xaa Gly
        195                 200                 205

Xaa Leu Xaa Xaa Ser Asp Phe Xaa Xaa Phe Xaa Asp Arg
    210                 215                 220
```

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 167 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

```
Cys Gly Pro Gly Arg Gly Xaa Xaa Xaa Arg Arg Xaa Xaa Xaa Pro Lys
1               5                   10                  15

Xaa Leu Xaa Pro Leu Xaa Tyr Lys Gln Phe Xaa Pro Xaa Xaa Xaa Glu
            20                  25                  30

Xaa Thr Leu Gly Ala Ser Gly Xaa Xaa Glu Gly Xaa Xaa Xaa Arg Xaa
        35                  40                  45

Ser Glu Arg Phe Xaa Xaa Leu Thr Pro Asn Tyr Asn Pro Asp Ile Ile
    50                  55                  60

Phe Lys Asp Glu Glu Asn Xaa Gly Ala Asp Arg Leu Met Thr Xaa Arg
65                  70                  75                  80

Cys Lys Xaa Xaa Xaa Asn Xaa Leu Ala Ile Ser Val Met Asn Xaa Trp
                85                  90                  95

Pro Gly Val Xaa Leu Arg Val Thr Glu Gly Xaa Asp Glu Asp Gly His
            100                 105                 110

His Xaa Xaa Xaa Ser Leu His Tyr Glu Gly Arg Ala Xaa Asp Ile Thr
        115                 120                 125

Thr Ser Asp Arg Asp Xaa Xaa Lys Tyr Gly Xaa Leu Xaa Arg Leu Ala
    130                 135                 140

Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr Glu Ser Xaa Xaa His Xaa
145                 150                 155                 160

His Xaa Ser Val Lys Xaa Xaa
                165
```